

**Remarks:**

The May 11, 2004 Official Action has been carefully considered. In view of the amendment submitted herewith and these remarks, favorable reconsideration and allowance of this application are respectfully requested.

At the outset, it is noted that a shortened statutory response period of three (3) months was set in the May 11, 2004 Official Action. The initial due date for response, therefore, was August 11, 2004. A petition for a three (3) month extension of the response period is presented with this amendment and request for reconsideration, which is being filed before the expiration of the three (3) month extension period, as November 11, 2004 fell on a federal holiday.

Claims 1-15, 19-21, 29-43, and 59-61 are currently under consideration in this application, claims 13-15, 33 and 36 having been rejoined for examination on the merits.

In the May 11, 2004 Official Action, the examiner objects to claim 2, based on the recitation of an acronym, namely, FGF8. The meaning of the recited acronym has been inserted in claim 2 in accordance with this amendment, thereby overcoming this ground of objection.

Turning to the substantive aspects of the May 11, 2004 Official Action, claims 1-15, 19-21, 29-31, 37-43 and 59-61 stand newly rejected for allegedly failing to comply with the written description provision of 35 U.S.C. §112, first paragraph.

In this regard, the examiner states that the rejected claims embrace embodiments where the factor or factors are delivered apart from the astrocytes in some form, and that it cannot be concluded that applicants had in their possession the relatively isolated factor or factors required to practice the claimed invention in the broadly recited genus of methods embraced by the rejected claims.

Claims 29-43, which are directed to various screening methods, have been newly rejected as allegedly failing to satisfy the enablement provision of 35 U.S.C. §112, first paragraph.

After considering the so-called "Wands factors" (In re Wands, 8 U.S.P.Q.2nd 1400 (Fed. Cir. 1988)), the examiner concludes that it would have required undue, unpredictable experimentation to identify the factors referred to in the rejected claims, and/or their receptors using the recited methods.

Claims 1-3, 6-15 and 41-43 have also been rejected as allegedly failing to satisfy the enablement provision of 35 U.S.C. §112, first paragraph. The examiner acknowledges that the present specification is enabling for methods of inducing a dopaminergic neuronal fate in a neuronal stem cell or neuronal progenitor cell where the neural precursor cell expresses *Nurr1* above basal levels and where the neural precursor is contacted with one or more factors secreted from a Type 1 astrocyte of the ventral mesencephalon while *in vitro* co-culture with the Type 1 astrocyte. In the examiner's view, however, the specification does not reasonably provide enablement for treatment methods in which 1) the induction of dopaminergic cell fate is induced *in vivo*, 2) inducted dopaminergic cells are provided to a subject in *ex vivo* methods of treatment or 3) the factor or factors responsible for induction of dopaminergic cell fate are used as part of a pharmaceutical formulation for treatment of a subject in need thereof. Once again taking the "Wands factors" into account, the examiner concludes that the quantity of experimentation necessary to carry out the claimed methods is high and of an unpredictable nature, as the skilled artisan purportedly could not rely on the prior art or the present specification to teach how to use the claimed methods to necessarily achieve a therapeutic effect in an individual suffering a neurological disorder of the brain. According to the examiner, the experimentation required would have been of trial-and-error nature, at best, and would have been undue, unpredictable experimentation. The examiner further contends in this regard that the specification does not reasonably provide enablement for any method of treatment based upon the induced dopaminergic cells.

Claims 19-21, 29-33, 36-43 and 59-61 have also been newly rejected for allegedly failing to particularly point out and distinctly claim the subject matter which applicants regard as the invention. Specifically, the following claims are deemed vague and indefinite in view of the recitations: "in accordance with" (claims 19 and 59); "obtainable" (claim 29); "which further comprises isolating and/or purifying and/or cloning said receptor or receptors" (claim 30); "screening for a factor or factors in . . . expressing Nurrl above basal levels" (claims 29[sic], 32 and 34); "which comprises differential expression screening" (claim 36); and "wherein a factor or factors able to induce . . . in isolated and/or purified form" (claim 37). Furthermore, claim 29 is considered vague and indefinite for the additional reason that there is purportedly no clear nexus between the stated outcome of the claimed method and the actual method steps recited therein.

Finally, claims 19-21 and 59-61 have been further rejected under 35 U.S.C. §102(e) as allegedly anticipated by either U.S. Patent No. 6,284,539 to Bowen et al. (rejection maintained from July 16, 2003 Official Action) or U.S. Patent No. 5,981,165 to Weiss et al. (new ground of rejection).

The foregoing objection and rejections constitute all of the grounds set forth in the May 11, 2004 Official Action for refusing the present application.

In accordance with the present amendment, claim 1 has been amended to include the subject matter of claim 4. Claim 4 has accordingly been canceled, and a consequential amendment has been made to change the dependency of claim 5.

Claim 2 has been amended to spell out the acronym "FGF8" as previously noted. A similar amendment has been made to claim 8.

Claims 29 and 31 have been amended to recite that the factor or factors "are obtained from Type 1 astrocytes of the ventral mesencephalon".

Claim 30 has been amended to require cloning of the

gene or genes that encode(s) the receptor(s) referred to therein.

Claim 32 has been amended to reflect that the screening method is to identify a factor or factors which, either alone or in combination, induce a dopaminergic fate in a neural stem cell or progenitor cell expressing Nurr1 above basal levels and that the occurrence of the binding referred to therein identifies the molecules being screened as containing said factor or factors. Similarly, claim 34 has been amended to reflect that the screening method is to identify a factor or factors which, either alone or in combination, induce a dopaminergic fate in a neural stem cell or progenitor cell expressing Nurr1 above basal levels, and that the occurrence of the differentiation referred to therein identifies the molecules being screened as containing said factor or factors.

The subject matter of claim 36 is now set forth in new claim 69, and claim 36 has accordingly been canceled.

Claim 37 has been amended to recite that the factor or factors referred to therein are provided in the method of screening in isolated and/or purified form.

Claim 40 has been amended so as to be dependent on claim 39, rather than claim 38.

Withdrawn claims 23-28, 48-54, and 58 have been canceled pursuant to this amendment. However, the subject matter of claim 48, in part, is now set forth in new claim 62, which depends from claim 1. New claims 63-68 correspond to withdrawn claims 49-54, but depend, directly or indirectly, from new claim 62.

Claims 19-21 and 58-61 have also been canceled pursuant to this amendment, thereby rendering moot the various rejections of those claims set forth in the May 11, 2004 Official Action.

The cancellation of claims 19-21, 23-28, 48-54, 58-61, and each of them, pursuant to this amendment is without prejudice to applicants' right to file one or more continuing applications with respect to the subject matter of the canceled claims (as well as claim 1 in its original form), in accordance with 35

U.S.C. §§120 or 121, as the case may be.

No new matter has been introduced into this application by reason of the present amendment.

For the reasons set forth below, the 35 U.S.C. §112, first paragraph rejection of claims 1-15, 29, 31 and 37-43 based on alleged lack of written description, the 35 U.S.C. §112, first paragraph rejection of claims 29-43 and claims 1-3, 6-15 and 41-43 based on alleged inadequate enablement and the 35 U.S.C. §112, second paragraph rejection of claims 29-33 and 36-43, based on alleged indefiniteness, as set forth in the May 11, 2004 Official Action, either lack merit or cannot be maintained in view of the present amendment, or both. Those grounds of rejection are, therefore, respectfully traversed.

**A. Claims 1-15, 29-31 And 37-43 Fully  
Comply With The Written Description Provision  
Of 35 U.S.C. §112, First Paragraph**

The relevant inquiry in determining compliance with the written description provision of 35 U.S.C. §112, first paragraph is whether the specification reasonably conveys to a person having ordinary skill in the relevant art that applicants, at the time the application was filed, had possession of the claimed subject matter. In re Kaslow, 217 U.S.P.Q. 1089 (Fed. Cir. 1983).

Furthermore, the examiner has the initial burden of presenting evidence or reasons why a person of ordinary skill in the art would not recognize in applicants' specification disclosure a description of the invention defined by the claims. Ex Parte Sorenson, 3 U.S.P.Q.2nd 1462 (Bd. Apps. 1987).

The lack of written description rejection cannot be maintained with respect to claims 1-15, 29-31 and 37-43 as presently amended.

Claim 1, as amended, is directed to embodiments of applicants' method in which the neural stem or neural progenitor cell is co-cultured with a Type 1 astrocyte of the ventral

mesencephalon, thereby contacting the cell *in vitro* with one or more factors secreted from said Type 1 Astrocyte of the ventral mesencephalon, so as to produce dopaminergic neurons. These embodiments of applicants' invention are clearly described in the present application as filed. The examiner has acknowledged as much at page 6 of the May 11, 2004 Official Action. Thus, the lack of written description rejection set forth in the May 11, 2004 Official Action is inapplicable to claims 1-15 and now amended.

Claims 29-31 and 37-43 are directed to screening methods which are not, and should not be limited to situations in which the factor(s) are provided in co-culture with Type 1 astrocytes. The receptor screening method of claims 29 and 30 is described in the present specification at page 23. The factor screening method of claims 31 and 37-43 is described in the present specification at pages 20-23. It has long been held that a rejection based on the description requirement is improper where (as here) the specification describes the invention in the same terms as the claims. In re Bowen, 181 U.S.P.Q. 48 (CCPA 1978). In view of the clear correspondence between the present specification and the subject matter of claims 29-31 and 37-43, as noted above, claims 29-31 and 37-43 fully comply with the written description provision of 35 U.S.C. §112, first paragraph.

For all of the foregoing reasons, it is clear that in the present case, the examiner has failed to satisfy the PTO's burden of proof with respect to the lack of written description rejection, as applied to the subject matter of amended claims 1-15, 29-31 and 37-43. Accordingly, this ground of rejection is untenable and should be withdrawn.

**B. Claims 1-3, 6-15 And 29-43 Satisfy The Enablement Provision Of 35 U.S.C. §112, First Paragraph**

1. Claims 1-3 and 6-10

As previously noted, claim 1 has been amended to recite that the neural stem or neural progenitor cell is co-cultured

with a Type 1 astrocyte of the ventral mesencephalon, thereby contacting the cell in vitro with one or more factors secreted from the Type 1 astrocyte of the ventral mesencephalon, so as to produce dopaminergic neurons. Accordingly, claim 1, as well as claims 2, 3 and 6-10, which depend therefrom, are commensurate in scope with the enablement provided in applicants' specification, as acknowledged by the examiner at page 11 of the May 11, 2004 Official Action. The 35 U.S.C. §112, first paragraph rejection of claims 1-3 and 6-10 for alleged failure to comply with the enablement provision of 35 U.S.C. §112, first paragraph, should, therefore, be withdrawn.

2. Claims 11-15 and 38-43

Claims 11-15 further define the method of claim 1 by calling for formulating a dopaminergic neuron produced by the method of claim 1 into a composition (claim 11), including a pharmaceutically acceptable excipient in the composition (claim 12), administering the composition to an individual (claim 13), implanting the dopaminergic neurons into the brain of the individual (claim 14), and further characterizing the individual as having Parkinson's disease (claim 15). Similarly, claims 38-43 further define the factor screening method of claim 31 with respect to its formulation into a composition and administration to an individual, which may be via implantation to the brain of the individual, and include an individual having Parkinson's disease. As such, claims 11-15 and 38-43 do not encompass induction of the dopaminergic fate of neural stem or neural progenitor cells *in vivo* or *in situ*. Moreover, claims 11-15 and 38-43 do not encompass gene therapy methods and the examiner's comments addressed to such therapy, i.e. by reference to the Verma article and Hsich's review, are clearly irrelevant to the patentability of applicants' claimed invention.

Nor do claims 11-15 and 38-43 cover use of the factor(s) that induce dopaminergic cell fate, in the absence of a neural stem or neural progenitor cell, for administration to an individual.

Applicants respectfully take exception to the examiner's position, insofar as it is based on alleged inadequate enablement with regard to the use of a neural stem or neural progenitor cell induced to a dopaminergic fate *in vitro*, for implantation of the dopaminergic neuron in an individual, including those suffering from Parkinson's disease. The concerns expressed by the examiner in this regard are greatly exaggerated. First, there have been numerous publications demonstrating that progenitor cells transplanted in the adult brain of Parkinson's disease patients survive in the absence of immunosuppression therapy, thus indicating that it may not be required. See, for example, Freed et al, J. Neurol., 250[Suppl.3]: III/44-III/46 (2003) (copy attached). On the other hand, if immunosuppression would be necessary, there are several options available. It is noteworthy in this connection that all current kidney, liver, heart, lung and pancreas transplantation is performed with heterologous donors. The same immunosuppression criteria that apply for such approaches would be applicable to applicants' treatment methods.

Regarding the notion that results obtained in animal models are not predictive of activity in humans, there is considerable data reported in the literature which contradicts that view in the field of this invention. Research in animal models over a period of thirty (30) years has translated remarkably well into the clinic. See, for example, Von Bohlen und Halbach et al., Prog. Neurobiol., 73 (3): 151-77 (2004); and Akerud et al., J. Neurosci. 21: 8108-18 (2001) (copies attached). The examiner has cited no evidence that supports his position in this regard.

In response to the examiner's underlined statement bridging pages 19 and 20 of the May 11, 2004 Official Action, it is clearly shown in the present specification that progenitor cells processed in accordance with the present invention release dopamine and it is known that in rodents, the levels of dopamine in the rodent *corpus striatum* correlate directly with the motor



activity of the rodent, and the integration of the graft in the host striatum improves the motor performance. Similarly, in humans, the number of grafted cells correlate with Fluoro-DOPA uptake, and both Fluoro-DOPA uptake and the number of dopaminergic neurons in the graft correlate with therapeutic effects in Parkinson's disease patients. Moreover, therapeutic effects in addition to those of dopamine release are also obtained by long term functional integration of the grafted cells in Parkinson's disease patients. Thus, data in the literature indicate that the engraftment of dopaminergic neurons in the adult host striatum, such as the Nurrl-neuronal stem cell-derived dopaminergic neurons, is the main criterion that has to be met in order to achieve a therapeutic effect.

In summary, in the case of Parkinson's disease, there is ample evidence indicating a good correlation between *in vitro* and *in vivo* models and between *in vivo* models and therapeutic response in strategies that restore dopamine levels.

For the above-stated reasons, the 35 U.S.C. §112, first paragraph rejection of claims 11-15 and 38-43 based on alleged inadequate enablement is improper and should be withdrawn.

3. Claims 29-37

Claims 29-31 are directed to a method for screening for a receptor or receptors for a factor or factors that, alone or in combination, induce a dopaminergic fate in a neural stem or progenitor cell expressing Nurrl above basal levels. It is the Examiner's position that the specification fails to provide "significant guidance as to how one would definitively identify a putative receptor." The Examiner also contends that the prior art fails to provide significant guidance for practicing the claimed method.

Applicants respectfully disagree. From page 21, line 33 through page 26, line 28, of the present specification Applicants have provided substantial guidance as to how to perform various means of screening in order to perform the instantly claimed method. With respect to screening for a

receptor or receptors of the factor or factors secreted from Type I astrocytes, the specification discloses at page 23, lines 7-17 that cells expressing above basal levels of Nurr1 can be compared to normal cells. As noted at page 22, lines 22-28, this comparison or screening method can be performed by "any known method for analyzing a phenotypic difference between cells and may be at the DNA, mRNA, cDNA, or polypeptide level." Notably, the specification further teaches at page 21, lines 26-33 that the interaction between, e.g., the factor and the receptor, "may be determined by any number of techniques in the art" including "labeling either one with a detectable label and bringing it into contact with the other which may have been immobilized on a solid support, e.g. by using an antibody bound to a solid support." Applicants submit that the above teachings are sufficient, in view of the techniques widely known and used by skilled artisans at the time of the invention, to enable one skilled in the art to perform the instantly claimed methods. Indeed, it is a well settled premise in patent law that a patent need not teach, and preferably omits, what is well known in the art. *Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 231 USPQ 81, 94 (Fed. Cir. 1986).

Prior to the effective filing date of the instant applicant, methods for detecting unknown receptors or binding partners of proteins were generally known in the art. For example, Evander et al. (J. Virol. (1997) 71:2449-2456) (copy attached) teach a method for the identification of the receptor of the human papillomavirus. The method entailed radiolabeling cells which bind the human papillomavirus and cells which do not bind the human papillomavirus (see page 2449 and page 21, lines 26-33 of the instant specification). The radiolabeled cellular extracts were contacted with human papillomavirus virus-like particles (VLPs) which were bound by an antibody to a solid support (page 2450 and page 21, lines 26-33 of the instant specification). The bound radiolabeled proteins were then analyzed by gel electrophoresis to determine which radiolabeled proteins were present only on cells known to bind human

papillomavirus (Fig. 2A). Thus, Applicants submit that a skilled artisan could readily radiolabel the factors secreted from Type I astrocytes, contact the radiolabeled factors to neural stem or progenitor cells optionally immobilized on a solid support, optionally crosslink the radiolabeled factors to the receptors on the neural stem or progenitor cells to promote stability, and employ gel electrophoresis to separate and then compare proteins from cells with and without expressing Nurrl above basal levels. Radiolabeled protein bands present only with the cells expressing Nurrl above basal levels would be indicative of the factor or factors bound to the sought receptor or receptors. To identify the receptor(s), unique protein band(s) could be excised from the gel and analyzed by mass spectrometry, a technique commonly employed at the time of the instant invention to identify the sequence of a protein (see, e.g., Ausubel et al., ed. (Current Protocols in Molecular Biology (1998) John Wiley & Sons, Inc., 10.22.1-10.22.24, at 10.22.3) (copy attached).

As another example, Ranganathan et al. (J. Biol. Chem. (1999) 274:5557-5563) (copy attached) teach the identification of the binding partner of the low density lipoprotein receptor related protein 2/megalin (LRP-2). Briefly, seminal vesicle fluid was separated by gel electrophoresis and then placed on a solid support by Western blotting (see, e.g., Fig. 2). Radiolabeled LRP-2 was then added to the solid support in the presence or absence of an inhibitor. Protein bands that bound the radiolabeled protein in the absence of the inhibitor were then excised and identified by mass spectrometry.

Applicants submit that a skilled artisan, in view of prior art teachings such as Ranganathan et al., and the guidance of the instant specification, could arrive at various means to identify the receptor of the factor secreted by Type 1 astrocytes. For example, a skilled artisan may mount the secreted proteins from Type 1 astrocytes on a solid support by Western blotting as performed by Ranganathan et al. (see page 21,

lines 26-33 of the instant specification). The skilled artisan could then add radiolabeled membrane fractions from cells with and without expressing Nurrl above basal levels (see page 21, lines 19-33 of the instant specification). The two samples could then be compared and protein bands unique to the cells expressing Nurrl above basal levels could be excised. The excised protein bands could be identified by mass spectrometry, as stated hereinabove. Alternatively, the skilled artisan could place neural stem or progenitor cell surface proteins on the solid support as performed by Ranganathan et al. and contact the bound proteins with radiolabeled secreted proteins from Type I astrocytes. Protein bands unique to cells expressing above basal levels of Nurrl would then be excised and sequenced by mass spectrometry, thereby identifying the sought receptor.

Thus, Applicants submit that the specification, in view of techniques well known in the art at the time of the invention, is enabling for the instantly claimed methods.

Claims 31-37 are directed to methods of screening for a factor or factors that, alone or in combination, induce dopaminergic fate in neural stem or progenitor cells expressing Nurrl above basal levels. It is the Examiner's position that the specification only provides the "observation that at least one such factor appears to be secretable" and that the factor is "highly labile." Applicants submit, however, that the specification clearly enables a skilled artisan to practice the instantly claimed methods. As stated hereinabove, the specification provides a significant number of ways to screen for the factor or factors at page 21, line 33 through page 26, line 28 of the instant specification. Furthermore, the specification teaches that the factor or factors responsible for inducing dopaminergic fate are clearly secretable based on the barrier experiments (see, e.g., page 38, lines 17-24). Applicants also submit that the teaching that at least one of the factor or factors is labile, in fact, further enables a skilled artisan to perform the instantly claimed method. Indeed, apprised of this

information, the skilled artisan would employ measures routinely used in the art to help stabilize the factor or factors, such as employing reduced temperatures and introducing protease inhibitors. Inasmuch as Applicants have provided significant guidance and numerous means by which the skilled artisan can identify the factor secreted by Type 1 astrocytes, Applicants submit that a skilled artisan is fully enabled to practice the instantly claimed methods.

In summary, for the reasons set forth above the 35 U.S.C. §112, first paragraph rejection of claims 1-3, 6-15 and 29-43 based on alleged inadequate enablement is untenable and should be withdrawn

**C. Claims 29-33 And 36-43 Fully Comply**  
**With The Definiteness Provision Of 35**

**U.S.C. §112, Second Paragraph**

The relevant inquiry in determining compliance with the definiteness provision of 35 U.S.C. §112, second paragraph, is whether the claim in question sets out and circumscribes a particular area with a sufficient degree of precision and particularity, such that the metes and bounds of the claimed invention are reasonably clear. In re Moore, 169 U.S.P.Q. 236 (CCPA 1971).

Regarding the allegation that the language of claim 29 lacks clarity, claim 29 has been amended to call for "one or more factors obtained from a Type 1 astrocyte . . . .".

Notwithstanding the examiner's assertion to the contrary, claim 29 cannot reasonably be held to be vague and indefinite with regard to the nexus between the stated outcome of the method and actual method steps recited therein. It is the comparison of the cells which do or do not express Nurr-1 above basal levels that leads to identification of the receptor(s). Hence, there is a clear nexus between the stated outcome of the claimed method and the actual method steps recited therein.

Claim 30 has been amended, as noted above, to include

language to the effect that it is the gene(s) encoding the receptor(s) that is/are cloned. As for the difference between "isolating" and "purifying", the difference lies in the scope of the respective terms. Thus, one can perform an isolation of a substance without purifying it, e.g. by a partitioning or fractioning process, but one cannot purify a substance, i.e. render it free from undesired elements, without isolating it. Although there is some overlap in the two (2) terms, each one is sufficiently clear to apprise others of the boundaries beyond which experimentation and invention are undertaken at the risk of infringing claim 30. The same applies to the recitation of "isolated" and "purified" in claim 37.

Claims 29, 32 and 34 are improperly treated together in the statement of rejection appearing at page 23 of the May 11, 2004 Official Action. Claim 29 does not include the allegedly objectionable phrase. Claims 32 and 34 have been amended to call for identification of the factor(s).

Claim 36 has been rewritten as new claim 69 and indicates the manner in which differential expression screening is used in the method of claim 33.

Claim 37 has been amended to include language to the effect that the factor(s) is/are provided in the screening method in isolated and/or purified form.

In summary, applicant's position with respect to the rejection of claims 29-32 and 36-43 based on 35 U.S.C. §112, second paragraph, is that any person of ordinary skill in the art, having applicants' disclosure and claims before him or her, would be apprised to a reasonable degree of certainty as to the exact subject matter encompassed within claims 29-33 and 36-43. Nothing more is required under 35 U.S.C. §112, second paragraph. The §112, second paragraph, rejection of claims 29-33 and 36-43 set forth in the May 11, 2004 Official Action should accordingly be withdrawn.

It is noted in passing that, in relation to the 35 U.S.C. §102(e) rejections of claims 19-21 and 59-61 in view of

Bowen et al. and Weiss et al., neither of the cited references teaches a method involving Nurrl and Type 1 astrocytes of the ventral mesencephalon, as required in claims 19-21 and 59-61. Nevertheless, the cancellation of claims 19-21 and 59-61 renders these rejections moot.

In view of the present amendment and the foregoing remarks it is respectfully requested that the objection and rejections set forth in the May 11, 2004 Official Action be withdrawn and that this application be passed to issue and such action is earnestly solicited.

Respectfully submitted,

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Enclosures:

- Freed et al, J. Neurol., 250[Suppl.3]: III/44-III/46 (2003)
- Von Bohlen und Halbach et al., Prog. Neurobil., 73 (3): 151-77 (2004)
- Akerud et al., J. Neurosci. 21: 8108-18 (2001)
- Evander et al. (J. Virol. (1997) 71:2449-2456)
- Ausubel et al., ed. (Current Protocols in Molecular Biology, John Wiley & Sons, Inc., 10.22.1-10.22.24, at 10.22.3 (1998)
- Ranganathan et al. (J. Biol. Chem. (1999) 274:5557-5563)

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## Do patients with Parkinson's disease benefit from embryonic dopamine cell transplantation?

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□ **Abstract** Embryonic dopamine cell transplants survive in nearly all patients regardless of age and without immunosuppression. Transplants can improve Parkinson "off" symptoms up to the best effects of L-dopa observed preoperatively. They cannot improve the "best on" state. Transplants appear to survive indefinitely. In 10 to 15 % of patients, transplants can reproduce the dyskinetic effects of L-dopa even after discontinuing all L-dopa.

Neurotransplantation should be tried earlier in the clinical course of Parkinson's to see if earlier intervention can prevent progression of the disease, particularly the dyskinetic responses seen after long-term L-dopa treatment.

■ **Key words** putamen · positron emission tomography (PET) · fluorodopa · embryonic stem cells · double-blind · neurosurgery

For about 15 years, embryonic dopamine cell transplantation has been performed in humans [1–3]. Since that time, a number of open clinical trials have described benefits from transplantation. Interestingly, outcomes have been similar in these reports, despite the fact that many different experimental techniques have been used. The validity of these observations has been uncertain because of the small numbers of subjects, variable inclusion criteria, and a lack of controls.

The election of Bill Clinton as President of the United States in 1992 made it possible to design a controlled clinical trial with support from the National Institutes of Health. We joined with colleagues Stanley Fahn, MD, at Columbia University and David Eidelberg, MD, of North Shore University Hospital to develop a double-blind, placebo-controlled clinical trial of neurotransplantation. Prior to this trial, no double-blind study had been performed in the field of neurosurgery. While there was opposition at the time [4], we had decided that valid conclusions could be drawn only from a large group of subjects who received an identical transplant operation while being compared with a control surgical group that underwent a surgical procedure identical to the transplant except for penetration of the brain. The patients

and the examining physicians would remain unaware of the procedure performed until after the breaking of the blind.

Our surgical methods evolved over several years. Human transplant research was helped enormously by preceding work done in rodents, and to a lesser extent, in monkeys. Before our first human transplant in 1988, rodent studies had shown that only dopamine neurons from early in development could survive and reinnervate brain (7 to 8 weeks' post conception in humans). Experiments also showed that immunosuppression was probably unnecessary for within-species allografts. This second point is critically important, since the long-term costs and complications of immunosuppression are difficult to justify for symptomatic treatment of Parkinson's disease. Because the putamen is the structure most linked to motor cortex and is the region most depleted of dopamine in idiopathic Parkinson's disease, bilateral implants into putamen have been our standard operation. We have performed transplants into putamen in 61 patients. Three patients also received tissue into caudate unilaterally and one also received tissue implants bilaterally into the region of the substantia nigra pars compacta. To reduce the number of needle passes into the



brain, we developed a novel transplant method with needle trajectories passing through the forehead above the frontal sinus and projecting through the long axis of putamen (35 to 40 mm), bilaterally. Four needle passes have been made, one dorsal and one ventral on each side of brain, separated by a distance of about 7 mm. Tissue has been kept in tissue culture for up to one month prior to transplant [5].

For the NIH study, mesencephalic dopamine neurons from four embryos were implanted, with strands of tissue from a single embryo used for a single needle track. For that study, 40 patients were recruited with 20 under age 60 and 20 over that age. The goal was to compare placebo with tissue implants in younger and older patients. No patient was immunosuppressed. After the blind was broken, sham surgery subjects had the option of returning for a tissue implant, and 14 of 20 did so, yielding a total of 34 transplant subjects in the trial.

Blinded reading of fluorodopa PET scans one year after transplant revealed transplant growth in 85% of patients, even without immunosuppression [5]. Growth was the same in both younger and older subjects. In the younger group, but not the older, objective measures showed improvement of UPDRS motor "off" scores of 34% and of Schwab and England "off" scores of 31% while sham surgery patients did not improve. There were no changes in the "best on" scores. Subsequent analysis has shown that it is not patient age, but the preoperative response to L-dopa that predicts the magnitude of neurologic change after transplant. As a group, the older patients had shown lower and more variable effects of L-dopa before the transplant surgery. Older subjects who had a good response to L-dopa (> 60% improvement in motor scores) had the same response to transplant as younger subjects. Reduced effectiveness of L-dopa in some older patients may reflect more global brain disease as a cause of their Parkinsonian symptoms. Transplants cannot influence those non-dopaminergic systems.

Patients who received transplants after prior sham surgery were compared to patients transplanted under the blinded protocol. Clinical improvements observed over the first year were identical in the two groups, further demonstrating that there were no measurable placebo effects. There were no changes in cognitive measures in either younger or older patients.

The importance of the double-blind design cannot be overstated. Other double-blind trials performed after ours and testing transplants of human embryonic tissue and of bovine embryonic tissue have failed to show objective clinical improvement, despite promising results in small open trials. For this field to progress, double-blind comparisons must continue to be performed.

Beginning with our first human experiments, we have found that most patients who show improved motor scores also develop dyskinesias, usually resolving af-

ter reduction of L-dopa and other dopamine agonists [1, 3, 5]. Subsequently, others have made similar observations. In about 10% of cases, deep brain stimulating electrodes implanted in STN or GPi may be necessary to control persistent dyskinesias.

What is an "ideal" transplant outcome? The most desirable would be for a patient to regain a normal neurologic exam after discontinuing all medications. Our experience indicates that such an outcome will only occur in patients who have a normal neurologic exam while on L-dopa prior to transplant. In patients with advanced Parkinson's disease, a realistic outcome is for a transplant to reduce the severity of the "off" state and provide about 50% of the maximum benefit of L-dopa. L-dopa and other drugs still must be administered to fine-tune the "on" state.

An intriguing and open question at this time is whether transplants can prevent the progression of Parkinson's disease. We have observed that patients followed for up to five years after transplant have had no progression of symptoms [6]. It is possible that transplants performed early in the course of Parkinson's disease might prevent the appearance of the complications of drug therapy, such as drug-induced dyskinesias. This hypothesis will require clinical testing.

A report of a second double-blind trial of human embryonic dopamine cells has been published [7]. This study was designed to test the effect of tissue dose, with sham operations compared to transplant of tissue from two embryos or six to eight embryos per patient. The blind was maintained for 24 months. PET scanning showed greater transplant outgrowth at 12 months from the larger dose, while the smaller dose grafts had progressive growth that caught up with the larger dose grafts by 24 months. There was no change in patients who received sham operations. Despite robust graft survival, this study failed to see any improvement in UPDRS motor "off" scores in the transplanted group compared to controls. Their results were markedly different from ours, since our overall transplant group and the group age 60 or under had significant improvement in UPDRS motor "off" scores and in the Schwab and England "off" scores. From the results available so far, the most likely explanation for the differences in outcome is that the patients in the Olanow study were not sufficiently responsive to L-dopa on entry into the study. Surprisingly, they found patients developed severe dyskinesias without a period of improvement. We found that four patients of 34 transplanted developed dyskinesias following one to two years of dramatic clinical improvement. In our study, the dyskinesias after transplant mimicked the L-dopa-induced dyskinesias in the same patients prior to transplant.

In the individual patient, transplant development is uncertain, primarily because the vast majority of dopamine cells die from apoptotic programmed cell

death in the days after transplantation. Pretreatment with neurotrophic factors can reduce this cell loss and may make transplantation effects more uniform. The biggest limitation to wide scale testing and application of neurotransplantation is the limited availability of hu-

man embryonic dopamine neurons. Laboratory production of large quantities of dopamine neurons from a stem cell source could solve this problem. Human embryonic stem cells may be successfully differentiated into dopamine neurons, perhaps in the near future [8].

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# Genes, proteins, and neurotoxins involved in Parkinson's disease

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## Abstract

Parkinson's disease (PD) is a common neurodegenerative disorder. The etiology of PD is likely due to combinations of environmental and genetic factors. In addition to the loss of neurons, including dopaminergic neurons in the substantia nigra pars compacta, a further morphologic hallmark of PD is the presence of Lewy bodies and Lewy neurites. The formation of these proteinaceous inclusions involves interaction of several proteins, including  $\alpha$ -synuclein, synphilin-1, parkin and UCH-L1. Animal models allow to get insight into the mechanisms of several symptoms of PD, allow investigating new therapeutic strategies and, in addition, provide an indispensable tool for basic research. In animals PD does not arise spontaneously, thus, characteristic and specific functional changes have to be mimicked by application of neurotoxic agents or by genetic manipulations. In this review we will focus on genes and gene loci involved in PD, the functions of proteins involved in the formation of cytoplasmatic inclusions, their interactions, and their possible role in PD. In addition, we will review the current animal models of PD.

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**Abbreviations:** 6-OHDA, 6-hydroxydopamine; AD, Alzheimer's disease; AR-JP, autosomal recessive juvenile parkinsonism; CDCrel-1, cell division control-related protein 1; CNS, central nervous system; DAergic, dopaminergic; DA, dopamine; DLB, dementia with Lewy bodies; DOPAL, 3,4-dihydroxyphenylacetaldehyde; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; ir, immunoreactivity; LB, Lewy body; IMAN, lateral magnocellular nucleus of the anterior neostriatum; LN, Lewy neurites; Mn-EBDC, manganese ethylene-bis-dithiocarbamate; MPP<sup>+</sup>, 1-methyl-4-phenyl-2,3-dihydropyridium ion; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MSA, multiple system atrophy; NMDA, *N*-methyl-D-aspartate; NO, nitric oxide; Pael-R, parkin-associated endothelin receptor-like receptor; PD, Parkinson's disease; ROS, reactive oxygen species; SN, substantia nigra; SNpc, substantia nigra pars compacta; T<sub>1</sub>Clo, 1-trichloromethyl-1,2,3,4-tetrahydro-beta-carboline; TH, tyrosine hydroxylase; UCH, ubiquitin carboxyl-terminal hydrolase; UPP, ubiquitin-proteasome pathway; VMAT, vesicular monoamine transporter; VTA, ventral tegmental area

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## 1. Introduction

Parkinson's disease (PD) was originally described by James Parkinson in 1817. PD is one of the most common neurodegenerative disorder affecting about 4 million people worldwide (Bushnell and Martin, 1999). PD is characterized by a trias of cardinal symptoms, namely bradykinesia (slowed movement), resting tremor, and rigidity. Normal motor function depends on the regulated synthesis and release of dopamine (DA) by neurons projecting from the substantia nigra to the striatum (Chase et al., 1998). Degeneration of the DAergic neurons located in the substantia nigra pars compacta (SNpc) and a subsequent loss of DAergic nerve terminals in the striatum are responsible for most of the movement disturbances (Chase et al., 1998; Nagatsu et al., 2000). There is a gradual decline in DAergic neurons of the SNpc during aging, which is accompanied by a reduction of the striatal DA content. In idiopathic PD, however, the symptoms become apparent when about 70% of the striatal DA and about 50% of the nigral DAergic neurons are lost (Dunnett and Bjorklund, 1999). Therefore, symptomatic management of PD with L-DOPA and other DAergic drugs dominates current therapy and is highly effective in managing early stages of the disorder. However, long-term treatment often goes along with a loss of drug efficacy, the onset of dyskinesias and the occurrence of psychosis (Jenner, 2003a). Therefore, non-DAergic agents, which are effective in animal models of PD (as in the MPTP model, see Section 12.2), e.g. adenosine A2 antagonists (Richardson et al., 1997), are discussed as potential drugs in the therapy of motor dysfunctions in PD, without inducing dyskinesia (Bibbiani et al., 2003; Jenner, 2003a). Indeed, patient studies indicate that KW6002, an adenosine A2 antagonists, together with L-DOPA, may prolong the duration of antiParkinsonian action of L-DOPA and reduces severity of dyskinesia (Kase et al., 2003; Bara-Jimenez et al., 2003; Chase et al., 2003).

Further pathological features of PD are dystrophic neurites termed "Lewy neurites" and eosinophilic inclusions, known as Lewy bodies (LBs) (Forno, 1996). In PD, LBs are

found in many regions of the nervous system, including the substantia nigra, locus coeruleus, cortex, limbic areas, hypothalamus, nucleus basalis, cranial nerve motor nuclei, and central and peripheral divisions of the autonomic nervous system (Takahashi and Wakabayashi, 2001). Thus, the presence of LBs in association with nerve cell loss in the substantia nigra and various other regions of the nervous system is a diagnostic hallmark of PD. Since  $\alpha$ -synuclein is one of the major components of LBs (Iwatsubo, 2003), the process of LB formation can be divided into several stages (Wakabayashi et al., 1998; Takahashi and Wakabayashi, 2001).

- Stage 1: diffuse cytoplasmatic anti- $\alpha$ -synuclein staining.
- Stage 2: occurrence of irregularly shaped staining of moderate intensity.
- Stage 3: discrete staining corresponding to "pale bodies".
- Stage 4: ring-like staining of a typical LB with a central core and a surrounding halo.

Within the substantia nigra, neurons exhibiting stage 1 or stage 2 structures are more numerous in patients with a shorter disease duration than in those with a longer duration or in incidental, subclinical PD patients (Wakabayashi et al., 1998). The pale bodies, seen in stage 3 are considered to represent precursors of Lewy bodies or to represent a place of LB formation (Takahashi and Wakabayashi, 2001).

The underlying mechanisms of most cases of PD are still unknown, but, recently, specific genetic defects have been identified. In addition, also environmental factors may contribute to the disease. Epidemiologic studies have indicated that a number of factors that may increase the risk of developing PD (Tanner and Langston, 1990). Despite the fact that numerous exogenous toxins have been associated with the development of parkinsonism, no specific toxin has been found in the brain of PD patients. In many instances the parkinsonism seen in association with toxins is not that of typical Lewy body PD (Olanow and Tatton, 1999). Thus, the pathogenesis is thought to be multifactorial, deriving from environmental factors acting on genetically predisposed individuals with aging (Riess and Kruger, 1999). However,

## 2. Mechanisms of cell death

The mechanisms involved in the progressive degeneration of nigrostriatal DAergic neurons in PD, however, are of major interest in PD-research. Recently, particular attention has focused on several conceptually distinct mechanisms, including mitochondrial dysfunction, oxidative stress or excitotoxicity. It may be possible that they all interact and amplify each other (Dunnett and Bjorklund, 1999), leading to neuronal dysfunction, atrophy as well as cell death (Fig. 1).

The mitochondrion is the primary site for the generation of energy supply for the cell. The mitochondrial generation of energy is regulated by five respiratory chain complexes. Complex I controls the transfer of one electron from NADPH to the coenzyme Q and the transfer of two protons to the mitochondrial intermembrane space. These protons are then used by complex V to synthesize ATP from ADP (Alberts et al., 1987).

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graph TD; Excitotoxicity -.-> Mitochondrial_damage[Mitochondrial damage]; Mitochondrial_damage -.-> free_radicals[free radicals (ROS)]; free_radicals -.-> peroxynitrite; peroxynitrite -.-> CELL_DEATH[CELL DEATH]; CELL_DEATH -.-> nitric_oxide[nitric oxide]; nitric_oxide -.-> Excitotoxicity; nitric_oxide -.-> peroxynitrite; Mitochondrial_damage -.-> CELL_DEATH; Excitotoxicity -.-> influx_calcium[influx of calcium]; influx_calcium -.-> nitric_oxide;
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Mitochondrial dysfunction, however, might not exclusively be related to DAergic neurons of the SNpc but may be systematic, since mitochondrial dysfunction in PD is also observed in the striatum (Mizuno et al., 1989) and other tissues (Parker et al., 1989; Cardellach et al., 1993; Haas et al., 1995; Parker and Swerdlow, 1998).

## 2.2. Oxidative stress

Accumulating evidence supports the hypothesis that oxidative stress involving lipid peroxidation may contribute to the pathogenesis of PD (Yoshikawa, 1993). The brain depends mostly on mitochondrial energy supply which is associated with the production of highly reactive oxygen species (ROS). Ninety-five percent of the molecular oxygen is metabolized within the mitochondria by the electron-transport-chain so that mitochondria are highly exposed to oxidative stress which may damage distinct neuronal populations (Tritschler et al., 1994).

There are several potential sources of increased oxidative stress in PD, including mitochondrial dysfunction, increased free iron levels and impaired free radical defence mechanisms (Foley and Riederer, 2000). Oxygen radicals generated during respiration induce mitochondrial dysfunction, which accelerates the production of more deleterious species of oxygen. The latter step further increases mitochondrial malfunction, thus intensifying and perpetuating the cycle (Tritschler et al., 1994). Therefore, mitochondrial dysfunction and oxidative stress, either by impairments in the mechanism for scavenging ROS or by increased production of ROS, could contribute to cell death in PD. Under specific circumstances oxidative stress could develop in the substantia nigra, leading to cell damage and cell death. An increased DA turn-over, resulting in peroxide formation in excess (Olanow and Tatton, 1999), could induce oxidative stress as well as other factors (Fig. 2). The generated ROS lead to functional alteration in proteins, lipids and DNA. Lipid damage, in turn, causes loss of membrane integrity and decreased membrane-permeability to ions, which can promote excitotoxicity (Halliwell, 1992).

Although ROS levels cannot be measured directly, the assessment of their reaction products and the resultant damage in post-mortem tissues serve as indirect indices of their levels (Foley and Riederer, 2000). These evidences for increased oxidative stress in PD brains include widespread ROS-induced protein modification and increased membrane oxidation in the SNpc (Dexter et al., 1994a,b) as well as increased 8-hydroxy-2'-deoxyguanosine levels in the SNpc,

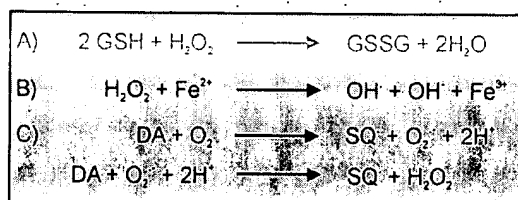


Fig. 2. Factors leading to oxidative stress in the SNpc: (A) a deficiency in glutathione (GSH), thereby diminishing the capability to clear H<sub>2</sub>O<sub>2</sub>; (B) an increase in reactive iron, which can promote OH• formation; (C) auto-oxidation of DA into toxic dopamine-quinone species (Lotharius and Brundin, 2002). GSSG: oxidized glutathione; SQ: dopamine-quinone species (adopted from Lotharius and Brundin, 2002).

indicative for ROS-mediated DNA damage (Alam et al., 1997).

Increased levels of nitric oxide (NO) can be neurotoxic. It is likely that most of the neurotoxic actions of NO are mediated by peroxynitrite (ONOO<sup>-</sup>), the reaction product from NO and superoxide anion (Dawson and Dawson, 1996). NO also inhibits the mitochondrial respiratory chain (Cleeter et al., 1994) and enhances MPP<sup>+</sup> inhibition of complex I. Nitric oxide synthase inhibitors protect against MPTP-induced neurotoxicity in rodents (Smith et al., 1994; Schulz et al., 1995) and prevent MPTP-induced parkinsonism in baboons (Hantraye et al., 1996).

## 2.3. Excitotoxicity

Damage due to excess glutamate, which changes the permeability of cells to calcium by acting on and through *N*-methyl-D-aspartate (NMDA) receptors, is considered to be involved in neurodegeneration. Glutamate-induced excitotoxicity is thought to represent a major mechanism in ischemia (Rothman and Olney, 1986; Ikonomidou and Turski, 1996) and in epilepsy (Olney et al., 1986) and might also play a role in PD. Massive activation of glutamate receptors can result in excessive rises in cytoplasmic Ca<sup>2+</sup> that are thought to underlie the fundamental processes ultimately leading to neuronal death (Mody and MacDonald, 1995). The calcium-dependent NMDA neurotoxicity is based on two mechanisms: (i) excessive nitric oxide formation (Jenner, 2003b) and (ii) mitochondrial dysfunction (Schinder et al., 1996).

- The extensive influx of calcium causes an activation of nitric oxide synthase, which converts L-arginine to citrulline and nitric oxide. Excess NO is in part responsible for glutamate neurotoxicity. It is likely that most of the neurotoxic actions of NO are mediated by peroxynitrite (ONOO<sup>-</sup>), the reaction product from NO and superoxide anion (Dawson and Dawson, 1996).
- The extensive Ca<sup>2+</sup> entry accompanying NMDA-receptor activation is largely accumulated by the intracellular mitochondria, with effects on mitochondrial membrane potential, ATP synthesis, glycolysis, reactive oxygen species generation, leading to failures in cytoplasmic Ca<sup>2+</sup> homeostasis and thus promoting cell death (Nicholls and Budd, 1998).

DAergic nigrostriatal neurons are rich in glutamate receptors and receive an extensive glutamatergic innervation from the cortex and the subthalamic nucleus (Olanow and Tatton, 1999). Since the substantia nigra receives rich glutamatergic inputs, it has been speculated that glutamate-induced excitotoxicity may be involved in cell death in PD (Dunnett and Bjorklund, 1999). In support of a role of glutamate mediated excitotoxicity in PD, it has been reported that NMDA antagonists protect against loss of DAergic neurons, resulting from MPTP treatment in rats (Turski et al., 1991) and primates (Crossman et al.,

1989; Greenamyre et al., 1994). Even so, data from human post-mortem studies indicate that the glutamatergic system may contribute to PD. Evaluation of glutamate binding sites in control and PD-diseased brains by autoradiography has revealed a reduction in NMDA-receptors as well as in  $\alpha$ -amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) binding sites, whereas the metabotropic binding sites seemed to be unaltered in the SNpc (Difazio et al., 1992). In addition, a decreased platelet glutamate uptake has been evidenced in patients with PD (Ferrarese et al., 2001). Several NMDA-receptor antagonists, including amantadine, are neuroprotective by inhibiting NMDA-receptor-dependent glutamate activity (Kornhuber et al., 1994). Moreover, there is evidence that amantadine ameliorates dyskinesia in PD patients (Godwin-Austen et al., 1970; Thomas et al., 2004). However, there are also reports describing that amantadine is not effective in the treatment of levodopa-induced dyskinesias in PD patients (Paci et al., 2001; Crosby et al., 2003).

The simplest explanation for the selective loss of DAergic nigrostriatal neurons in PD is that DA itself or a metabolite is neurotoxic. Indeed, it has been shown that 3,4-dihydroxyphenylacetaldehyde (DOPAL), a metabolite of DA, is a critical endogenous toxin which triggers DAergic neuron loss in PD (Burke, 2003).

### 3. Staging Parkinson's disease

The degeneration of SNpc neurons is considered to be one of the most important morphological hallmarks of PD (Gibb et al., 1991; Gibb, 1991; Damier et al., 1999). PD is not purely reflected by nigrostriatal degeneration. Thus, the formation of the very first Lewy neurites (LN) and LBs occurs in non-catecholaminergic neurons (Del Tredici et al., 2002). Thus, nigral and extranigral pathologies do not evolve simultaneously, but follow a predetermined sequence marked by characteristic changes in topographical extent. The pathological process extends beyond the substantia nigra and involves serotonergic, norepinephrinergic and acetylcholinergic neuronal populations. In addition to disturbances in the DAergic system, the imbalance in these neurotransmitters are also involved in the psychiatric, cognitive and autonomic dysfunctions seen in PD (Djaldetti and Melamed, 2002). It is believed that the lesions evolve in the course of PD sequentially, beginning at predisposed sites and extending from there in a predictable manner throughout the brain. The gradual ascent of this process has been grouped by Braak and coworkers (Braak et al., 2001, 2002, 2003) into six stages (Fig. 3).

- The occurrence of LBs and LNs in the dorsal motor nucleus of the vagal nerve characterizes stage 1 of PD.
- In stage 2 the lesions within the dorsal vagal region increase in severity, and raphe nuclei (nuclei raphe magnus, obscurus and pallidus), the reticular formation and the locus coeruleus become involved (Braak et al., 2001).

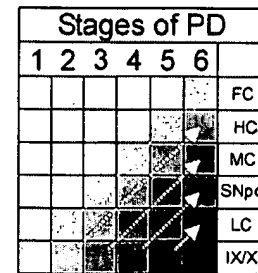


Fig. 3. Schematic representation of the topographic expansion of the lesions (from left to right) and the growing severity on the pathology (from top to bottom: stages 1–6), as indicated in different gray scales (adopted from Braak et al., 2003). IX/X: dorsal motor nucleus of the glossopharyngeal and vagal nerves; LC: locus coeruleus; SNpc: substantia nigra pars compacta; MC: mesocortex (anteromedial temporal area); HC: high order sensory association areas and prefrontal fields; FC: first-order sensory association areas, premotor areas and primary sensory and motor fields.

- Stage 3 is characterized by an involvement of the SNpc as well as of the pedunculopontine tegmental nucleus and of the magnocellular nuclei of the basal forebrain.

These first three stages are considered to belong to a pre-symptomatic phase of PD. The symptomatic phase of PD has also been classified in three stages (stages 4–6).

- In stage 4, the SNpc is severely affected, displaying a significant loss of neurons. Furthermore, the central nucleus of the amygdala, the temporal mesocortex and allocortex become involved.
- In the final stages 5 and 6, the neurodegeneration attains its fullest topographic extent. Pathological changes in the areas involved in the earlier stages become more severe and inclusion bodies appear in association areas of the neocortex, agranular and granular insular fields, the anterior cingulate cortex and prefrontal areas (stage 5). In the last stage (stage 6), nearly the entire neocortex is involved (Braak et al., 2002, 2003).

According to that view, these different stages indicate that the evaluation of the substantia nigra is not sufficient for the neuropathological evaluation of questionable PD cases, since the substantia nigra is not the first structure in the brain, in which PD-related abnormalities develop (Del Tredici et al., 2002). However, it should be mentioned that individuals that develop inclusions during the early stages (stages 1 and 2) did not suffer from PD. Whether these patients had presymptomatic PD and would finally develop the full phenotype of the disease is still unknown.

The presence of LBs is the diagnostic hallmark of PD; they can be recognized by their eosinophilic cores and unstained halos. These eosinophilic bodies are found in specific regions of the nervous system. However, they differ in frequency, size, shape, and structure (Gibb et al., 1991), although all LBs have a common composition. They are composed of eosinophilic intracellular proteinaceous inclusions containing lipids, neurofilaments and related proteins, e.g. synphilin-1, ubiquitin and ubiquitin-pathway-related

enzymes (Chung et al., 2001a; Lotharius and Brundin, 2002). The major component of LBs is an aggregated form of the presynaptic protein  $\alpha$ -synuclein. Although it is still unknown why  $\alpha$ -synuclein gradually transforms into virtually insoluble LBs or LNs, it is the best marker available for the visualization of PD-related lesions (Spillantini et al., 1998). Thus, for neuropathological diagnosis  $\alpha$ -synuclein-immunopositive LNs and LBs are essential (Braak et al., 2003). However, abnormal accumulation of  $\alpha$ -synuclein does not only occur in PD, but also in other diseases, like dementia with Lewy bodies (DLB), multiple system atrophy (MSA), and the Lewy body variant of Alzheimer's disease (Giasson et al., 2000; Jellinger, 2003). These diseases have therefore been termed  $\alpha$ -synucleinopathies (Galvin et al., 2001a; Marti et al., 2003).

#### 4. Alpha-synuclein

Alpha-synuclein belongs to a family of 15–20 kDa proteins (Kahle et al., 2002), currently consisting of three different members:  $\alpha$ -synuclein,  $\beta$ -synuclein and  $\gamma$ -synuclein (Clayton and George, 1998). All three different synucleins are expressed in the brain of humans and rodents (Giasson et al., 2001; Galvin et al., 2001b; Li et al., 2002).

Alpha-synuclein was first identified in the electric ray *Torpedo californica* (Maroteaux et al., 1988). The rat  $\alpha$ -synuclein cDNA has been cloned in the same year (Maroteaux et al., 1988), while the mouse cDNA was not cloned before 1998 (Hong et al., 1998). Human  $\alpha$ -synuclein was first identified as the precursor protein (NACP) for the non-amyloid beta component (NAC) of Alzheimer's disease (AD) amyloid plaques (Ueda et al., 1993). NAC has been isolated in the SDS-insoluble fraction of AD brains. NAC is located in the central part of the  $\alpha$ -synuclein protein and extends from amino acid positions 61–95, although the amino terminus is not definitely determined. The human  $\alpha$ -synuclein/NACP gene (SNCA or PARK1) consists of six exons ranging in size from 42 to 1110 bp and is located on the long arm of the human chromosome 4q21–q23 (Polyméropoulos et al., 1996). The translation start codon ATG is encoded by exon 2 and the stop codon TAA is encoded by exon 6. The non-A $\beta$  component of Alzheimer's disease amyloid (NAC) is encoded by exon 4 (Xia et al., 2001). The human  $\alpha$ -synuclein gene encodes a small (140 amino acid) protein characterized by repetitive imperfect repeats (KTKEGV) distributed throughout most of the amino-terminal half of the polypeptide (Fig. 4), a hydrophobic middle region (NAC region), and an acidic carboxy-terminal region (Eliezer et al., 2001). The highly conserved amino-terminal repeat domain of  $\alpha$ -synuclein is thought to mediate lipid binding and dimerization (Jensen et al., 1997). In addition, this domain shares a common "natively unfolded" tertiary structure that has been implicated in protein-protein interactions (Weinreb et al., 1996; Davidson et al., 1998). Under physiological conditions

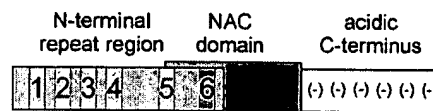


Fig. 4. Schematic representation of synucleins. The synucleins are composed of a N-terminal region with 5–6 imperfectly conserved repeats; the middle part of the synucleins is composed of the NAC domain; in less conserved C-terminus acidic amino acid chains are clustered. The imperfect KTKEGV repeats are numbered. The sixth repeat, which is missing in  $\beta$ -synuclein, is highlighted (adopted from Kahle et al., 2002).

in vitro,  $\alpha$ -synuclein is characterized by the lack of rigid well-defined structure, and thus belongs to the class of intrinsically unstructured proteins. Intriguingly,  $\alpha$ -synuclein is characterized by a remarkable conformational plasticity, adopting a series of different conformations depending on the environment (Uversky, 2003). For example,  $\alpha$ -synuclein may either stay substantially unfolded, or adopt an amyloidogenic partially folded conformation, or fold into alpha-helical or beta-structural species, both monomeric and oligomeric. Furthermore, it might form several morphologically different types of aggregates, including oligomers (spheres or doughnuts), amorphous aggregates, and or amyloid-like fibrils (Uversky, 2003).

The mouse  $\alpha$ -synuclein gene was mapped to the mouse chromosome 6 (Touchman et al., 2001). The intron/exon structure of the mouse and human  $\alpha$ -synuclein genes is highly conserved (Touchman et al., 2001) and the human and murine  $\alpha$ -synuclein protein sequences are 95.3% identical (Lavedan, 1998).

Alpha-synuclein is expressed throughout the brain at high levels, whereby the most prominent expression of synuclein mRNAs is in the hippocampus of rats (Maroteaux and Scheller, 1991) and mice (Hong et al., 1998). The protein seems to localize to nerve terminals, since no  $\alpha$ -synuclein-immunoreactive neuronal perikarya have been found in the rat hippocampus (Mori et al., 2002). Furthermore, within the rat brain,  $\alpha$ -synuclein-immunoreactivity is distributed with high intensity in nerve terminals of the caudate putamen and ventral pallidum (Li et al., 2002). Moreover, in the normal rat brain,  $\alpha$ -synuclein positive signals have been detected in the SNpc, but not in the perikarya of SNpc neurons (Li et al., 2002). In the brainstem,  $\alpha$ -synuclein was found to be present in the locus coeruleus and in the vagus nucleus, and in the oculomotor, facial, hypoglossal, accessory and ambiguous nuclei (Li et al., 2002). In each of these locations, with exception of the dorsal motor nucleus of the vagus, immunoreactivity was localized in the nerve terminals (Li et al., 2002). In contrast to these reports, using a monoclonal antibody (termed Syn-1) instead of polyclonal anti- $\alpha$ -synuclein antibodies, it has been shown that  $\alpha$ -synuclein is also present in the somata of specific neuronal populations in the rat brain, as, e.g. in the SNpc (Andringa et al., 2003).

The subcellular localization of the synucleins has not been established in detail, and suggested sites of action include the presynaptic terminals, the nuclear envelope and



the cytoplasm (Maroteaux and Scheller, 1991; Lavedan, 1998). Thus,  $\alpha$ -synuclein co-localizes with synaptophysin-immunoreactive presynaptic terminals, but the synaptic targets are unknown (Iwai et al., 1995). It further has been shown that under normal, non-pathological conditions,  $\alpha$ -synuclein binds to small unilamellar phospholipid vesicles containing acidic phospholipids, but not to vesicles with a net neutral charge (Davidson et al., 1998).

Reported binding targets for  $\alpha$ -synuclein include phospholipase D2 (Jenco et al., 1998), an enzyme that catalyzes the production of intracellular mediators, like phosphatidic acid (PA) and diacylglycerol (DAG). Furthermore,  $\alpha$ -synuclein is able to inhibit protein kinase C activity (Ostrerova et al., 1999). In addition, in cell cultures, using  $\alpha$ -synuclein transfected B103 neuroblastoma cells, it has been shown that  $\alpha$ -synuclein up-regulates the expression of calveolin-1 and down-regulates extracellular signal-regulated kinase (ERK) activity (Hashimoto et al., 2003). Since ERK plays a central role in various neuronal functions, including survival (Subramaniam et al., 2003), neuronal growth, synapse formation, synaptic plasticity and long-term potentiation (LTP) (Impey et al., 1999), modulation of the ERK pathway might be an important mechanism in normal  $\alpha$ -synuclein functions. In addition,  $\alpha$ -synuclein might be also be associated with axonal transport (Mizuno et al., 2001a). It is thought that under normal conditions,  $\alpha$ -synuclein may have a role in the modulation of synaptic vesicle turn-over and synaptic plasticity (Clayton and George, 1998). The idea that  $\alpha$ -synuclein has a role in synaptic plasticity originates from studies in song birds. The avian homologue of  $\alpha$ -synuclein is synelfin. Synelfin mRNA has been found to be up-regulated during a critical period of song learning in the lateral magnocellular nucleus of the anterior neostriatum (IMAN) of zebra finches *Taeniopygia guttata* (George et al., 1995). Interestingly, the synelfin protein is located in the presynaptic terminals from IMAN in the robust nucleus of the archistriatum (Jin and Clayton, 1997), another brain nucleus in birds, which is critically involved in song learning.

Since the mammalian  $\alpha$ -synuclein is found to be predominately expressed in areas of the adult CNS that display synaptic plasticity, including the cerebral cortex, the hippocampus, the amygdala and the olfactory bulb (Maroteaux and Scheller, 1991; Iwai et al., 1995), it is possible that  $\alpha$ -synuclein is also involved in synaptic plasticity in the mammalian brain. However,  $\alpha$ -synuclein knockout mice did not show any altered LTP in area CA1 of the hippocampus by using tetanic stimulation (Abeliovich et al., 2000).

Direct evidence for the involvement of  $\alpha$ -synuclein in PD was provided by genetic studies of patients with rare, dominantly inherited variances of this disorder. Several independent pathological mutations have been reported:

- a mutation in the  $\alpha$ -synuclein gene (G209A), leading to a change from alanine to threonine at the position

53 (Ala53Thr) in Italian-American and Greek families (Polymeropoulos et al., 1997);

- a mutation in the  $\alpha$ -synuclein gene (G88C), leading to a change from alanine to proline at the position 30 (Ala30Pro) in a family of German origin (Kruger et al., 1998); and
- a mutation in the  $\alpha$ -synuclein gene (E46K) has recently been discovered in a Spanish family with autosomal dominant parkinsonism (Zarranz et al., 2004).

These mutant proteins, especially the Ala53Thy mutated  $\alpha$ -synuclein, display the tendency to form fibrils in vitro (Conway et al., 1998). Moreover, genomic triplication of the  $\alpha$ -synuclein gene has recently been associated with familial PD, where the disease is caused by overexpression of  $\alpha$ -synuclein (Singleton et al., 2003, 2004; Bradbury, 2003; Farrer et al., 2004).

Neither wild-type  $\alpha$ -synuclein nor mutated  $\alpha$ -synuclein fold into structured, globular forms in vitro (Weinreb et al., 1996; Conway et al., 1998). The transformation of  $\alpha$ -synuclein from its unfolded structure to a folded stage involves changes in its conformation and its quaternary structure (Goldberg and Lansbury, 2000). This change causes the abnormal deposits of aggregated  $\alpha$ -synuclein. There is no evidence for a stable, structured  $\alpha$ -synuclein monomer, i.e. the beta-sheet conformation is not long-lived in the absence of oligomerization (Rochet and Lansbury, 2000). As shown recently, the inhibition of the beta-sheet conformation in the absence of oligomerization seems to be due to the long N-terminal repeat domain of  $\alpha$ -synuclein (Kessler et al., 2003).

The abnormal aggregation of  $\alpha$ -synuclein forms the major filamentous component of LBs and LNs (Spillantini et al., 1998). Thus, a morphological hallmark of LBs is their  $\alpha$ -synuclein-immunoreactivity (Takahashi and Wakabayashi, 2001). Moreover, it was shown that not only neurons, but also glial cells, both astrocytes and oligodendrocytes, were also affected by  $\alpha$ -synuclein pathology (Takahashi and Wakabayashi, 2001).

At high concentrations, wild-type  $\alpha$ -synuclein can self-aggregate in solution to form fibrils and discrete spherical assemblies; this process is accelerated in mutated forms of  $\alpha$ -synuclein (Conway et al., 1998). Interestingly, several pesticides, including rotenone and paraquat, can induce a conformational change in  $\alpha$ -synuclein and, thus, accelerate the rate of  $\alpha$ -synuclein fibrils formation in vitro (Uversky et al., 2001). However, it is currently still unknown what or which factors induce the change from the soluble to the aggregated form of  $\alpha$ -synuclein, which can be found in LBs and in LNs. Within the inclusion bodies  $\alpha$ -synuclein is present in nitrated (Giasson et al., 2000) as well as in hyperphosphorylated forms (Neumann et al., 2002). Based on this, it has been speculated that phosphorylation of  $\alpha$ -synuclein at its serine residue Ser129 may be critically involved in synucleopathy (Fujiwara et al., 2002). Using antibodies, raised against the phosphorylated  $\alpha$ -synuclein, a staining pattern

can be observed in the human diseased brain (Saito et al., 2003), which resembles the stages of LB formation as proposed by Wakabayashi.

The molecular mechanism by which abnormal  $\alpha$ -synuclein oligomers cause dysfunction and death of DAergic neurons in the SNpc is still unknown. However, it has been confirmed by immunoelectron microscopy that  $\alpha$ -synuclein co-localizes with tyrosine hydroxylase (TH), the rate-limiting enzyme of DA biosynthesis (Perez et al., 2002). In cell-free systems, a dose-dependent inhibition of TH by  $\alpha$ -synuclein has been observed (Perez et al., 2002). To measure the impact of  $\alpha$ -synuclein on TH in DAergic cells, cells have been transfected with  $\alpha$ -synuclein. Overexpression of  $\alpha$ -synuclein did not significantly alter TH protein levels in these stably transfected cells. However, overexpressing cell lines revealed significantly reduced TH activity and a corresponding reduction in DA synthesis (Perez et al., 2002). Since the reduction in cellular DA levels were not due to increased DA catabolism or DA efflux, this suggests that  $\alpha$ -synuclein may play a role in the regulation of DA biosynthesis, acting to reduce the activity of TH.

Under normal conditions,  $\alpha$ -synuclein might have neuroprotective properties. At nanomolar concentrations  $\alpha$ -synuclein protects neurons against oxidative stress and excitotoxicity through the PI3/Akt signaling pathway in primary neuronal cultures (Seo et al., 2002). In contrast, in high micromolar concentration  $\alpha$ -synuclein exerts neurotoxic effects on these cell cultures (Seo et al., 2002).

Overexpression of wild-type or mutant  $\alpha$ -synuclein (Ala53Tyr; Ala30Pro) in the human cell-line SH-SY5Y led to increased ROS levels. Furthermore, the cells overexpressing the different  $\alpha$ -synucleins display reduced viability. These findings indicate that a functional link between  $\alpha$ -synuclein overexpression and apoptosis of neurons may exist, caused by a breakdown of the intracellular steady-state levels of ROS (Junn and Mouradian, 2002). Along this line, primary cultures of embryonic human mesencephalon, overexpressing wild-type or mutant human  $\alpha$ -synuclein (Ala53Thr) show a 27 or 49% loss of DAergic neurons, respectively (Zhou et al., 2002), with no impairment in viability of other cells in the culture (Zhou et al., 2002). Moreover, overexpression of  $\alpha$ -synuclein in the rat nigrostriatal system induces cellular and axonal atrophy, including  $\alpha$ -synuclein positive cytoplasmatic inclusions, which are accompanied by a loss of 30–80% of the nigral DAergic neurons and a 40–50% reduction in striatal DA (Kirik et al., 2002). These results indicate that nigral DAergic neurons are vulnerable to high levels of  $\alpha$ -synuclein, supporting a role for  $\alpha$ -synuclein in the pathogenesis of PD.

Unfortunately, these experiments do not allow to conclude whether high levels of soluble  $\alpha$ -synuclein or whether  $\alpha$ -synuclein positive inclusion bodies cause the cell death in the SN.

Although numerous studies on the aggregation properties of  $\alpha$ -synuclein have been reported, little is known about its normal degradation. With regard to proteolytic degra-

dation, it has been found that the serine protease neurosin (kallikrein-6) degrades  $\alpha$ -synuclein and co-localizes with pathological inclusions such as LBs and glial cytoplasmic inclusions (Iwata et al., 2003). In an in vitro study it has been shown that neurosin prevented  $\alpha$ -synuclein polymerization by reducing the amount of monomer and also by generating fragmented  $\alpha$ -synucleins that themselves inhibited polymerization. Moreover, down-regulation of neurosin caused accumulation of  $\alpha$ -synuclein within cultured cells, indicating that neurosin plays a role in  $\alpha$ -synuclein degradation (Iwata et al., 2003).

#### 4.1. Mouse models related to $\alpha$ -synuclein

Transgenic *Drosophila* flies (Feany and Bender, 2000; Auluck and Bonini, 2002; Pendleton et al., 2002) and rats overexpressing human  $\alpha$ -synuclein (Kirik et al., 2002; Klein et al., 2002; Lo et al., 2002) are available, however genetically manipulated mice represent one of the most widely used animal models of  $\alpha$ -synucleopathies. Like the normal human  $\alpha$ -synuclein and the two PD-related mutated forms of the protein, the mouse  $\alpha$ -synuclein adopts a “natively unfolded” or disordered structure (Rochet et al., 2000). The amino acid sequences of the mouse  $\alpha$ -synuclein and the human  $\alpha$ -synuclein, however, differ at seven positions. Mouse  $\alpha$ -synuclein, like the mutated human A53T  $\alpha$ -synuclein, contains a threonine residue at position 53 (Rochet et al., 2000). This structural difference in the mouse  $\alpha$ -synuclein should be kept in mind when interpreting the phenotypes of transgenic animal models of PD.

In a specific sub-strain of C57BL/6J mice, a chromosomal deletion of the  $\alpha$ -synuclein locus has been detected. It has been proposed to name this strain C57BL/6S (Specht and Schoepfer, 2001). These animals appear phenotypically normal and the expression of  $\beta$ -synuclein or  $\gamma$ -synuclein as well as the expression of synphilin-1 seemed to be unaffected (Specht and Schoepfer, 2001).

##### 4.1.1. Alpha-synuclein knockout mice

Mice with a targeted deletion of the  $\alpha$ -synuclein gene are viable and develop normally (Abeliovich et al., 2000). These knockout mice exhibit an intact brain architecture including a normal complement of DAergic neurons. However, they display a reduction in striatal DA and an attenuation of DA-dependent locomotor response following amphetamine treatment (Abeliovich et al., 2000), but they do not contain LBs (Goldberg and Lansbury, 2000).

MPTP is a neurotoxin that inhibits mitochondrial complex I and which was found to induce  $\alpha$ -synuclein aggregation in the SNpc (Kowall et al., 2000; Kuhn et al., 2003). Interestingly, the  $\alpha$ -synuclein knockout mice display a resistance to MPTP-induced degeneration of DAergic neurons and DA release. This resistance is not due to abnormalities of the DA transporter, which appears to function normally in  $\alpha$ -synuclein null mutant mice (Dauer et al., 2002).

The analysis of these  $\alpha$ -synuclein knockout mice gave the first hints to normal functions of  $\alpha$ -synuclein in the brain. Evidence was provided that  $\alpha$ -synuclein can play a role in synaptic vesicle recycling. Thus,  $\alpha$ -synuclein knockout mice exhibited significant impairments in the synaptic response to a prolonged train of repetitive stimulation capable of depleting docked as well as reserve pool vesicles. Moreover, the replenishment of the docked vesicles by reserve pool vesicles seemed to be slowed in these knockout mice. Thus, under normal conditions,  $\alpha$ -synuclein may be required for the genesis and/or maintenance of a subset of presynaptic vesicles, those in the “reserve” or “resting” pools (Cabin et al., 2002).

#### 4.1.2. Transgenic $\alpha$ -synuclein mice

The first transgenic mice that express human  $\alpha$ -synuclein were generated in 2000 (Masliah et al., 2000). These mice displayed a progressive accumulation of  $\alpha$ -synuclein and ubiquitin-immunoreactive inclusions in neurons in the neocortex, hippocampus, and substantia nigra. These alterations were associated with a loss of DAergic terminals in the basal ganglia and with motor impairments (Masliah et al., 2000). Since then, different transgenic  $\alpha$ -synuclein mice have been generated, overexpressing wild-type  $\alpha$ -synuclein, as well as mutated forms of human  $\alpha$ -synuclein. Among these transgenic  $\alpha$ -synuclein mice are those with a mutation in Ala53Thr (van der Putten et al., 2000; Giasson et al., 2002; Lee et al., 2002), Ala30Pro (Rathke-Hartlieb et al., 2001; Lee et al., 2002) or mutations in both positions (Richfield et al., 2002).

However, controversial results have been reported. Overexpression of wild-type  $\alpha$ -synuclein, driven by the platelet-derived growth factor  $\beta$  promoter, leads to selective nigrostriatal damages (Masliah et al., 2000). In addition, it has been reported that expression of A53T mutant human  $\alpha$ -synuclein under the control of mouse *Thy1* regulatory sequences in the nervous system of transgenic mice generated animals with neuronal  $\alpha$ -synucleinopathy (van der Putten et al., 2000). Despite a lack of transgene expression in DAergic neurons of the SNpc, the features were strikingly similar to those observed in human brains with LB pathology, since neuronal degeneration and motor defects have been found (van der Putten et al., 2000).

Mice overexpressing mutant A53T human  $\alpha$ -synuclein, under the control of the prion promoter (Giasson et al., 2002; Lee et al., 2002), showed behavioral and neuronal dysfunction accompanied by aggregate formation, but not in the substantia nigra (Giasson et al., 2002; Lee et al., 2002). In addition, mice that overexpress the mutated human Ala30Pro  $\alpha$ -synuclein under the control of the prion promoter, have not revealed a neuropathology so far (Lee et al., 2002).

Thus, in some of these transgenic models,  $\alpha$ -synuclein expression leads to neurodegeneration, but a selective loss of DAergic neurons in conjunction with abnormal  $\alpha$ -synuclein deposits in the substantia nigra has not been observed.

Hence, these transgenic animals may rather represent a model of  $\alpha$ -synucleopathies than models of PD (Orth and Tabrizi, 2003).

Recently, a double transgenic mouse has been generated that expresses in neuronal tissues human  $\alpha$ -synuclein as well as human beta-amyloid. The functional and morphological alterations in these double transgenic mice resembled the Lewy body variant of Alzheimer's disease. In addition, these mice revealed deficits in learning and memory, and showed prominent age-dependent degeneration of cholinergic neurons. These double mutant animals display higher numbers of  $\alpha$ -synuclein-immunoreactive neuronal inclusions, compared to single  $\alpha$ -synuclein transgenic mice. These data may therefore indicate that beta-amyloid may contribute to the development of LB diseases by promoting the aggregation of  $\alpha$ -synuclein (Masliah et al., 2001).

## 5. Synphilin-1

In search for a candidate protein-interaction partner of  $\alpha$ -synuclein, a novel protein has been identified, which was named synphilin-1 (Engelender et al., 1999). The human synphilin-1 gene *SNCAIP* has been mapped to chromosome 5q23.1–23.3. The open reading frame of this gene consists of 10 exons; intron 5 possesses a highly polymorphic GT repeat (Engelender et al., 2000). Interestingly, the sequence of synphilin-1 seemed to be largely conserved, since the mouse synphilin-1 shows extensive homology with its human counterpart, with greatest similarities in those regions that contain ankyrin-like motifs and the coiled-coil domain (O'Farrell et al., 2002). In addition, expression of mouse synphilin-1 across tissues is similar to its human counterpart (O'Farrell et al., 2002).

In non-pathological brain tissues, synphilin-1 co-immunoprecipitated with synaptic vesicles, indicating a strong association with these structures. Furthermore, in vitro binding experiments demonstrated that the N-terminus of synphilin-1 is robustly associated with synaptic vesicles and that this association is resistant to high salt concentrations but can be abolished by inclusion of  $\alpha$ -synuclein in the incubation medium (Ribeiro et al., 2002).

Furthermore it has been reported that synphilin-1 is phosphorylated by glycogen synthase kinase-3 $\beta$  in vitro, indicating a possible involvement in signal transduction and/or protein degradation (Tanji et al., 2003).

In young rats, synphilin-1 was found to be expressed in neuronal cell bodies. During development, synphilin-1 seems to migrate gradually into the neuropil. Thus, in adult animals, synphilin-1 is highly enriched in presynaptic nerve terminals (Ribeiro et al., 2002). The role of both,  $\alpha$ -synuclein and synphilin-1, under normal conditions has yet to be clarified (Takahashi and Wakabayashi, 2001). However, under pathological conditions, it is known that  $\alpha$ -synuclein and its interacting partner synphilin-1 are among constituent proteins in LBs. Synphilin-1 associates

with  $\alpha$ -synuclein and promotes the formation of cytosolic inclusions (Engelender et al., 1999). In cell cultures, LB-like inclusion bodies can be formed when  $\alpha$ -synuclein and synphilin-1 were co-expressed. In these model, it has been demonstrated that the C-terminus of  $\alpha$ -synuclein is closely associated with the C-terminus of synphilin-1 (Kawamata et al., 2001). Using yeast two-hybrid screening, a synphilin-1 interacting protein has been identified (Nagano et al., 2003). This protein, Siah-1 ubiquitin ligase, was found to interact with the N-terminus of synphilin-1 through its substrate-binding domain, and specifically ubiquitinate synphilin-1 via its RING finger domain. Thereby, Siah-1 facilitated synphilin-1 degradation via the ubiquitin-proteasome pathway more efficiently than parkin. However, Siah-1 did not facilitate ubiquitination and degradation of wild-type or mutant  $\alpha$ -synuclein (Nagano et al., 2003).

In humans, synphilin-1 is present in LBs of patients with PD (Wakabayashi et al., 2000). In pathological brains, synphilin-1 immunoreactivity has been found in glial cells, in a small percentage of cortical (Katsuse et al., 2003) and nigral LBs (Murray et al., 2003), and in most LB of the brainstem (Wakabayashi et al., 2002). In the latter report, it has been shown that LB display intense anti-synphilin-1 staining in their central cores, whereas their peripheral portions were only weakly stained or unstained (Wakabayashi et al., 2002).

Despite the fact that several intronic mutations in the synphilin-1 gene have been identified, no pathogenic mutations in the synphilin-1 gene in PD has been detected until yet (Bandopadhyay et al., 2001; Satoh and Kuroda, 2002). However, a C to T transition in position 1861 of the coding sequence of synphilin-1 has been described recently in two apparently sporadic PD patients (Marx et al., 2003). This mutation leads to an amino acid substitution from arginine to cysteine in position 621. In cell culture systems it has been observed that cells, expressing the mutated synphilin-1, have significantly reduced numbers of inclusions compared to cells expressing wild-type synphilin-1, when subjected to proteasomal inhibition (Marx et al., 2003).

## 6. UCH-L1

Ubiquitin carboxyl-terminal hydrolases (UCH) are deubiquitinating enzymes, which hydrolyze C-terminal esters and amides of ubiquitin, leading to the generation of monomeric ubiquitin, the active component of the eukaryotic ubiquitin-dependent proteolytic system (Larsen et al., 1998). Ubiquitin carboxyl-terminal hydrolase L1 (UCH-L1) is a neuron-specific ubiquitin recycling enzyme (Nishikawa et al., 2003). UCH-L1, first identified as neuron-specific protein PGP 9.5, is one of the most abundant proteins in the brain, comprising up to two percent of the total brain protein content (Wilkinson et al., 1989, 1992), which can also be found in LBs (Lowe et al., 1990).

Based on the abundance of UCH-L1 in the brain and its presence in LBs as well as its involvement in ubiquitin-dependent proteolytic pathways, it is thought that UCH-L1 is implicated in the pathogenesis of PD (Leroy et al., 1998). Evidence for a critical role of UCH-L1 arises from the identification of a mutation of the UCH-L1 gene in a family with PD (Leroy et al., 1998). This missense mutation was found in a German PD family that contains a change of isoleucine to a methionine in position 93 of the fourth exon (Leroy et al., 1998). In the course of a failed search for additional I93M mutants (Harhangi et al., 1999), a previously unrecognized polymorphism in the UCH-L1 gene (S18Y) was discovered and subsequently found to be linked to a decreased susceptibility to PD (Maraganore et al., 1999; Lincoln et al., 1999; Wintermeyer et al., 2000; Zhang et al., 2000a). However, to date, there is no explanation for the fact that the I93M mutation is found in PD, although the S18Y mutation seems to be linked to a decreased susceptibility to PD (Liu et al., 2002), at least at a young age (Elbaz et al., 2003). Thus, it is not unlikely that the I93M substitution in the UCH-L1 gene is not the disease-causing mutation for idiopathic PD (Shi and Tao, 2003).

A malfunction of UCH-L1 could impair the overall efficiency of proteasomal protein degradation and thereby lead to an increased accumulation of damaged or misfolded proteins causing neuronal cell death (Chung et al., 2001a). UCH-L1 catalyzes the hydrolysis of C-terminal ubiquityl esters and amides, whereby peptide-ubiquityl amides are the preferred substrates (Larsen et al., 1996, 1998). In this pathway, UCH-L1 recycles used ubiquitin molecules (Lotharius and Brundin, 2002). Furthermore, UCH-L1 has been shown to exhibit an additional second, dimerization-independent, ubiquityl ligase activity (Liu et al., 2002). It therefore can be speculated that the hydrolytic activity may be beneficial, while the ligase activity could be harmful, by leading to accumulation of  $\alpha$ -synuclein, which escapes degradation.

In the gad mouse (gracile axonal dystrophy; an autosomal recessive mutation), homozygous truncating mutations were identified in the murine homologue of the UCH-L1 gene (Saigoh et al., 1999). These mice exhibit a neurodegenerative phenotype (Saigoh et al., 1999), but they do not develop a Parkinsonian phenotype (Saigoh et al., 1999; Kurihara et al., 2001). Analyses of these mice will be beneficial to elucidate the role of UCH-L1 in more detail.

## 7. Parkin

The human parkin gene (PARK2) has been mapped to chromosome 6q25.2–q27. The gene contains 12 exons spanning over 1.5 Mb and encodes a protein of 465 amino acids with a molecular mass of approximately 52.000 M (Kitada et al., 1998). Both, the mouse and rat parkin gene have been cloned in 2000 (Gu et al., 2000; Kitada et al., 2000).

Immunohistochemical analysis showed that parkin is expressed in neurons, but also in glial cells and blood vessels

of the human, monkey (Zarate-Lagunes et al., 2001) and rat brain (Gu et al., 2000). Parkin has been detected in neuronal perikarya of the substantia nigra, the striatum, the hippocampal formation, the pallidal complex, the red nucleus, and the cerebellum in human and monkey brains (Zarate-Lagunes et al., 2001).

Parkin protein is also widely distributed in all subdivisions of the rodent brain (Gu et al., 2000; Stichel et al., 2000). Low levels have been found in the telencephalon and diencephalon, while the brain stem contains a large number of cells heavily expressing parkin in mice (Stichel et al., 2000), rats (Gu et al., 2000) and birds (Horowitz et al., 2001). DAergic cells of the SNpc of mice exhibit high levels of parkin mRNA but no parkin protein, while the striatum contains immunopositive profiles but no mRNA signals (Stichel et al., 2000). In contrast, in rats, parkin mRNA and protein have been detected in the SNpc (Horowitz et al., 1999).

Autosomal recessive juvenile parkinsonism (AR-JP), one of the most common familial forms of PD, is characterized by selective DAergic neural cell death and the absence of LBs. Onset of this disease can be as early as the first decade of life (Lucking et al., 2000), but for most cases the onset is later than what could be considered “juvenile”. In fact, onset is best described as before the age of 40, although some rare cases can have onset as late as the sixth decade of life (Klein et al., 2000). In 1998, it has been discovered that AR-JP is linked to mutations in a single gene, which appear to be responsible for the pathogenesis of AR-JP. The gene product was named “parkin” (Kitada et al., 1998).

Neuropathological examination demonstrates that neuronal loss and gliosis are restricted to the substantia nigra and locus coeruleus; a Lewy pathology was not observed (Mori et al., 1998). The absence of LBs in PD patients with parkin mutations suggests that parkin might be required for the formation of LBs (Zhang et al., 2000b; Chung et al., 2001a). Indeed, parkin antibodies label classical LBs in substantia nigra sections in PD, which is not caused by parkin mutations (Schlossmacher et al., 2002). In these cases, in approximately 90% LBs parkin co-localizes with  $\alpha$ -synuclein (Schlossmacher et al., 2002).

Interestingly, a wide variety of mutations in the parkin gene, including exon deletions, duplications, and point mutations, result in autosomal recessive early-onset parkinsonism (Periquet et al., 2001). Thus, 12 different parkin mutations were found in 10 families from Europe and Brazil (Rawal et al., 2003). However, not all of these parkin mutations lead to early-onset parkinsonism. Thus, it has been shown that heterozygous mutations, especially those located in exon 7, act as susceptibility alleles for late-onset forms of PD (Oliveira et al., 2003). Moreover, not all mutations in the parkin gene lead to an absence of LBs. Thus, in case of a proband of a kindred with an exon 7 R275W substitution, in addition to the exon 3 deletion, LB pathology typical of idiopathic PD has been observed at autopsy (Farrer et al., 2001). This suggests that compound heterozygous parkin mutations

and loss of parkin protein may lead to early-onset parkinsonism with LB pathology, while a hemizygous mutation may confer increased susceptibility to typical PD (Farrer et al., 2001).

Parkin is a RING-finger-containing protein and was identified as an E3 ubiquitin-protein ligase (Imai et al., 2000). Thus, it is a component of the ubiquitin system, which is an important adenosine triphosphate-dependent protein degradation machinery (Mizuno et al., 2001b). Parkin is thought to act as a ubiquitin-protein ligase collaborating with the ubiquitin-conjugating enzyme (E2) that belongs to the cognate class of UbcH7 (Shimura et al., 2000) or UbcH8 (Tanaka et al., 2001). Parkin binds to the human E2 ubiquitin-conjugating enzyme 8 (UbcH8) through its C-terminal RING-finger and reveals ubiquitin-protein ligase activity in the presence of UbcH8 (Zhang et al., 2000b). Similar to other E3 RING-finger proteins (Joazeiro and Weissman, 2000), the RING-finger region of parkin confers the binding site for E2s (Giasson and Lee, 2001). Thus, mutations in parkin can disrupt the interaction with E2s, and it can also impair substrate recognition (Giasson and Lee, 2001). Therefore, mutations in parkin disrupt the E3 ubiquitin-protein ligase activity (Imai et al., 2000; Zhang et al., 2000b; Chung et al., 2001a,b) and thereby abolish the ubiquitin-protein ligase activity. In addition, parkin has been found to represent a tubulin-binding protein, as well as a microtubule-associated protein. Its ability to enhance the ubiquitination and degradation of misfolded tubulins may play a significant role in protecting neurons from toxins that cause PD (Ren et al., 2003).

Mutations in the parkin gene highlight that ubiquitin-mediated proteolysis may play an important role in the pathobiology of PD (Giasson and Lee, 2001). It is thought that the function of parkin in the ubiquitination pathway is to target misfolded proteins for degradation, as parkin protects against neurotoxicity induced by unfolded protein stress (Imai et al., 2000; Chung et al., 2001a; Lotharius and Brundin, 2002). Along this line, it was found that unfolded protein stress induces up-regulation of both the mRNA and protein level of parkin. Furthermore, overexpression of parkin specifically suppressed unfolded protein stress-induced cell death (Imai et al., 2000). In addition, parkin is able to suppress  $\alpha$ -synuclein-induced toxicity in cell culture systems (Oluwatosisin-Chigbu et al., 2003).

Since parkin is localized to the microsomal fractions, as well as to the cytosol—and Golgi fraction (Shimura et al., 1999), an endoplasmic reticulum (ER) associated protein degradation appears conceivable. Thus, mutation of the parkin gene could result in accumulation of misfolded proteins in the ER (Chung et al., 2001a). Indeed, parkin can assist in the removal of misfolded proteins in the ER, by ubiquitinating a homologue of the endothelin receptor type B, renamed “Pael-R” (parkin-associated endothelin receptor-like receptor). Parkin specifically ubiquitinates this receptor in the presence of ubiquitin-conjugating enzymes resident in the ER, and promotes the degradation of insol-

uble Pael-R. Thereby, cell death can be suppressed, that was originally induced by this receptor (Imai et al., 2001). Accumulation of Pael-R in the ER of DAergic neurons leads to neurodegeneration (Imai et al., 2002). Consistent with this function of parkin, the insoluble form of Pael-R has been found to be accumulated in the brains of AR-JP patients (Imai et al., 2001), suggesting that accumulation of unfolded Pael-R may lead to selective death of DAergic neurons (Takahashi and Imai, 2003).

Parkin also interacts with the synaptic vesicle-associated protein, cell division control-related protein (CDCrel-1). Parkin ubiquitinates and promotes the degradation of CDCrel-1 but familial parkin mutants (Q311 stop and T415N) are defective in CDCrel-1 degradation (Zhang et al., 2000b). However, the septin CDCrel-1 seemed to be dispensable for normal development (Peng et al., 2002). Thus, homozygous CDCrel-1 null mice appear normal with respect to synaptic properties and hippocampal neuron growth in vitro (Peng et al., 2002). Interestingly, viral vector-mediated overexpression of the parkin target protein CDCrel-1 in the substantia nigra of rats leads to dopamine-dependent neurodegeneration. This neurodegeneration is manifested by a progressive loss of nigral DAergic neurons and a decline of striatal dopamine levels (Dong et al., 2003).

More recently, a PDZ domain containing the scaffolding protein CASK/Lin2 has been shown to interact with the PDZ binding motif of parkin. A network of PDZ-interacting proteins has potential to form a complex net of molecules that surround parkin and regulate its subcellular localization and function (Dev et al., 2003). However, to date these interactions have not been investigated in detail.

### 7.1. Parkin knockout mice

Recently, parkin  $-/-$  mice have been generated (Goldberg et al., 2003). These mice are viable and display an increase in the extracellular dopamine concentration in the striatum. In contrast to the substantial loss of nigral neurons, characteristic for human PD, the number of dopaminergic cells in the substantia nigra of these mice is not altered (Goldberg et al., 2003), indicating that, at least in mice, parkin is not essential for the survival of nigral neurons. However, the mice exhibit deficits in behavioral paradigms, are sensitive to dysfunctions of the nigrostriatal pathway, but do not show obvious behavioral alteration in the rotarod test (Goldberg et al., 2003).

## 8. Linking $\alpha$ -synuclein, synphilin-1, parkin and UCH-L1

Abnormal accumulations of  $\alpha$ -synuclein can be found in LBs. Thereby, synphilin-1 seems to represent an  $\alpha$ -synuclein interaction partner. The neuronal expression patterns of  $\alpha$ -synuclein and parkin mRNAs are highly overlapping in the brain, suggesting that these two proteins may

play a role in the pathophysiology of PD (Solano et al., 2000). Indeed, parkin has been shown to ubiquitinate LB-like inclusion bodies (Chung et al., 2001a) and to protect against the toxicity associated with  $\alpha$ -synuclein (Petrucelli et al., 2002). Normal wild-type  $\alpha$ -synuclein does not interact with parkin directly (Shimura et al., 2001). Parkin seems to interact with glycosylated  $\alpha$ -synuclein only. In this case  $\alpha$ -synuclein is ubiquitinated by parkin (Shimura et al., 2001). In contrast to wild-type parkin, mutant parkin, associated with autosomal recessive PD, failed to bind glycosylated  $\alpha$ -synuclein and accumulates in its non-ubiquitinated form in parkin-deficient PD brains (Shimura et al., 2001).

Because parkin did not interact with (or ubiquitinate) non-glycosylated  $\alpha$ -synuclein, it has been speculated that other proteins, accumulated in LBs, could interact with parkin or could be ubiquitinated by parkin. Synphilin-1 associates with  $\alpha$ -synuclein and promotes the formation of cytosolic inclusions (Engelender et al., 1999). Moreover, it was found that parkin interacts with (or ubiquitinates) the  $\alpha$ -synuclein interacting protein synphilin-1 (Chung et al., 2001b). Ubiquitin-positive LB-like inclusions are formed when  $\alpha$ -synuclein, synphilin-1 and parkin are co-expressed.

Dearrangements in parkin function as well as mutations in UCH-L1 fit with the notion that dearrangements in the ubiquitin-proteasomal pathway (UPP) may play important roles in the demise of DAergic neurons in PD. Thus, mutations in parkin disrupt the ubiquitination of synphilin-1 and thus abolish the formation of ubiquitin-positive inclusions. Interestingly, cells co-transfected with synphilin-1 and  $\alpha$ -synuclein could form ubiquitinated inclusion bodies in the presence of parkin (Chung et al., 2001b).

Beside parkin, UCH-L1 is also involved in the UPP. The UPP is the main cellular pathway for disposal of abnormal proteins (Larsen et al., 1998). The selective degradation of many short-living proteins in eukaryotic cells is carried out by the ubiquitin system. In this pathway, proteins are targeted for degradation by covalent ligation of multiple ubiquitin molecules (Hershko and Ciechanover, 1998). Misfolded proteins that were tagged by ubiquitin chains were targeted to the proteosomes for destruction (Larsen et al., 1998). The degradation, catalyzed by ubiquitin carboxy-terminal hydrolases (UCH), is mediated by 26S proteosomes and includes the release of free and reusable ubiquitin (Ciechanover, 2001).

Ubiquitin-mediated degradation of regulatory proteins plays an important role in the control of cell-cycle progression, signal transduction, transcriptional regulation, receptor down-regulation, and endocytosis. Abnormalities in ubiquitin-mediated processes have been shown to cause pathological conditions, including malignant transformation (Hershko and Ciechanover, 1998). Moreover, the UPP can be overwhelmed in several neurodegenerative diseases. This results in the accumulation of toxic proteins and the formation of inclusions, which seem to be associated with

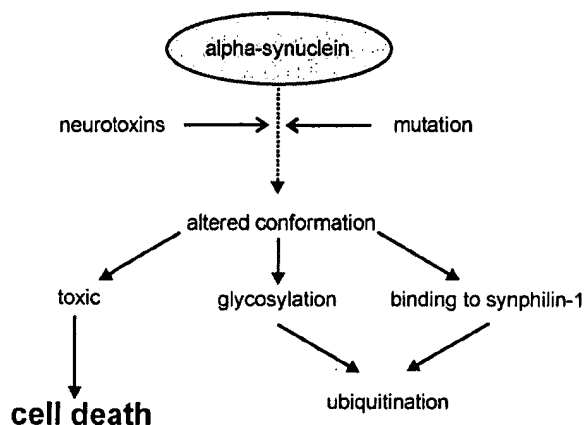


Fig. 5. Misfolded or mutated protein is thought to be neurotoxic. Either binding to synphilin-1 or glycosylation of alpha-synuclein allows binding of parkin. This binding subsequently allows ubiquitination and subsequent degradation by the ubiquitin system. Whether alpha-synuclein is degraded within the inclusion bodies is still unknown (Ciechanover, 2001). When the ubiquitin system is inactivated (e.g. by mutation in parkin) or failed to remove enough mutated or misfolded  $\alpha$ -synuclein, neurodegeneration might be caused.

neuronal dysfunction and cell death (Chung et al., 2001a). Along this line, not only parkin mutation have been identified in familial PD, but also mutations in UCH L-1 have been described in some PD cases (Leroy et al., 1998). The UCHs constitute a large family of de-ubiquitinating enzymes that can cleave polymeric ubiquitin into monomers. These enzymes may reduce the ubiquitin-tagged degradation of specific proteins, and they can be important for the regeneration of free and reusable ubiquitin following protein degradation (Hershko and Ciechanover, 1998). Aberrations in the UPP may cause aggregation of abnormal proteins as it is known for the pathogenesis of PD (Polymeropoulos, 2000). This suggests that failure of the UPP in the degradation of abnormal proteins may lead to nigral degeneration. Indeed, there is evidence to suggest that impaired protein clearance can induce cell death of DAergic ventral mesencephalic neurons (McNaught et al., 2002).

The finding that not all  $\alpha$ -synuclein positive inclusions contain ubiquitin (Goedert, 2001) indicated that ubiquitination of  $\alpha$ -synuclein is not a prerequisite for inclusion formation. Indeed, ubiquitination of  $\alpha$ -synuclein is not essential for inclusion formation: instead,  $\alpha$ -synuclein can be ubiquitinated either directly or indirectly within the inclusions (Sampathu et al., 2003). Misfolded  $\alpha$ -synuclein, which escapes ubiquitination, however, may lead to the death of DAergic neurons (Fig. 5).

It should also be mentioned that aside from the proteosomal degradation,  $\alpha$ -synuclein can also be degraded by autophagy. A role for autophagy is supported by the presence of  $\alpha$ -synuclein in organelles with the ultrastructural features of autophagic vesicles (Webb et al., 2003), but to date it is not clear to which extent autophagy plays a role in the degradation of misfolded  $\alpha$ -synuclein.

## 9. Dornin

In 2001, a novel gene, Dornin (a double RING-finger protein), was cloned from human spinal cord. The Dornin mRNA transcript is 4.4 kb and is expressed ubiquitously in many organs, including the CNS (Niwa et al., 2001). The gene encodes a protein of 838 amino acid, which contains two RING-finger motifs and an IBR (in between RING-fingers) motif at its N-terminus (Niwa et al., 2001). Dornin binds specifically to ubiquitin-conjugating enzymes UbcH7 and UbcH8 through the RING-finger/IBR domain (Niwa et al., 2001). It has been shown that dornin localizes to the ubiquitinated inclusions in PD but seemed not to bind  $\alpha$ -synuclein directly (Hishikawa et al., 2003). Thus, dornin ubiquitinates neither wild-type nor mutant  $\alpha$ -synuclein (Ito et al., 2003). Instead, dornin localizes to Lewy bodies and ubiquitylates synphilin-1 (Ito et al., 2003) indicating that dornin may be involved in the pathogenic process of PD and LB formation by ubiquitylation of synphilin-1.

## 10. Other gene loci involved in PD

In addition to PARK1 (representing the  $\alpha$ -synuclein gene), PARK2 (representing the parkin gene) and PARK5 (representing the UCH-L1 gene), other monogenic forms of PD have been described (Table 1).

The PARK3 locus has been mapped to chromosome 2p13 and has been identified in autosomal dominant PD cases (Lansbury and Brice, 2002). Recently, the human sideroflexin 5 gene (SFXN5), which maps to the critical PARK3 region, has been identified and characterized. Even so, the SFXN5 does not seem to correspond to PARK3 (Lockhart et al., 2002).

PARK4 has been mapped to 4p15 and has been found in autosomal dominant PD cases (Klein, 2001; Lansbury and Brice, 2002). However, it turned out recently that PARK4 does not represent a genetic mutation. In fact, it has been shown that an  $\alpha$ -synuclein locus triplication causes PD (Singleton et al., 2003). Thus, instead of the usual two

Table 1  
Characteristics of monogenic forms of parkinsonism

Designation	Locus	Gene	Inheritance
PARK1	4q21–22	$\alpha$ -Synuclein	AD
PARK2	6q25–27	Parkin	AR
PARK3	2p13	?	AD
PARK4 <sup>a</sup>	4p15	?	AD
PARK5	4p14	UCH-L1	?
PARK6	1p35–1p36	?	AR
PARK7	1p36	DJ-1	AR
PARK8	12p11.2–q13.1	?	AD

Mutation in single genes leading to PD (AD: autosomal dominant; AR: autosomal recessive; adopted from Lansbury and Brice, 2002).

<sup>a</sup> It has been shown recently that PARK4 is a triplication of a gene stretch containing  $\alpha$ -synuclein (Singleton et al., 2003).

copies of the  $\alpha$ -synuclein gene in the chromosome 4, four copies of the  $\alpha$ -synuclein gene have been found (see also Section 4).

The PARK6 locus was mapped to chromosome 1p35–p36 in different families with autosomal recessive parkinsonism (Valente et al., 2001, 2002).

PARK7 has been mapped to chromosome 1p36 in patients with an autosomal recessive early-onset parkinsonism (Van Duijn et al., 2001; Bonifati et al., 2002). It has also been found that mutations in DJ-1 are associated with autosomal recessive, early-onset PARK7 Parkinson's disease (Bonifati et al., 2003; Wilson et al., 2003). The function of the DJ-1 protein is unknown, but there is evidence to suggest that it is involved in the oxidative stress response and that loss of DJ-1 function leads to neurodegeneration (Bonifati et al., 2003). A further locus, PARK8, has been identified in a Japanese family with autosomal dominant parkinsonism. The PARK8 locus was mapped to a 13.6 cm interval on chromosome 12p11.2–q13p1 (Funayama et al., 2002).

## 11. Are Lewy bodies beneficial?

Although intracellular aggregates might be cytotoxic (Bence et al., 2001), some evidence has accumulated indicating that the presence of inclusion bodies might also be protective (Chung et al., 2001a). In PD, the prefibrillar  $\alpha$ -synuclein intermediate might be more toxic than the fibrillized  $\alpha$ -synuclein aggregates (Goldberg and Lansbury, 2000). Thus,  $\alpha$ -synuclein protofibrils, rather than the fibrils commonly found in LBs, might be the deleterious species (Lotharius and Brundin, 2002). Interestingly, DAergic neurons of the substantia nigra containing LBs appear to be "healthier", with regard to morphological and biochemical criteria, than neighboring neurons (Goldberg and Lansbury, 2000). It could be shown that in PD, the majority of SNpc neurons, which undergo apoptotic-like cell death, do not contain somal LBs. Possibly, they die before LB formation has occurred (Tompkins and Hill, 1997). Furthermore, striatal lesions with quinolinic acid induce death of about 25–30% of the DAergic neurons in the SN. In this model, synuclein was found to be expressed almost exclusively in morphologically healthy looking SNpc neurons, but not in neurons with apoptotic profiles (Kholodilov et al., 1999).

It has been reported that a combination of 1-methyl-4-phenylpyridinium ion (MPP<sup>+</sup>) or rotenone (two chemical compounds, which can be used for modeling PD in rodents, see Section 12.3.2) and proteasome inhibition cause the appearance of  $\alpha$ -synuclein positive inclusion bodies. Unexpectedly, however, proteasome inhibition blocked MPP<sup>+</sup>—or rotenone-induced dopaminergic neuronal death (Sawada et al., 2004). This indicates that the proteasome mediates DAergic neuronal degeneration and that its inhibition cause  $\alpha$ -synuclein inclusions. According to these results,  $\alpha$ -synuclein-inclusion body formation is not predictive for DAergic cell death.

Along this line, a recent publication also indicated that  $\alpha$ -synuclein-positive inclusion bodies may even be protective (Manning-Bog et al., 2003). Mice exposed to the neurotoxin paraquat (see also Section 12.3.2) showed marked degeneration of nigrostriatal neurons along with the formation of  $\alpha$ -synuclein-containing intraneuronal deposits. Mice overexpressing  $\alpha$ -synuclein, either the human wild-type or the Ala53Thr mutant form of the protein, displayed paraquat-induced protein aggregates but were completely protected against neurodegeneration (Manning-Bog et al., 2003).

Thus, LBs might have a protective role (Goldberg and Lansbury, 2000). Therefore, it has been speculated that the inclusions may sequester toxic species and/or divert  $\alpha$ -synuclein from toxic assembly pathways (Goldberg and Lansbury, 2000). The finding that parkin is an E3 ligase that can protect cells from unfolded protein stress, and that mutations in parkin lead to PD (Kitada et al., 1998), which is not accompanied by LBs (Shimura et al., 1999, 2000), also point in this direction. Thus, when parkin is mutated, it is no longer able to ubiquitinate misfolded proteins, which then cannot be degraded by the ubiquitin pathway. Under these circumstances, the substrates for parkin are toxic. The resulting cytosolic accumulation of proteins that would otherwise have been ubiquitinated and sequestered in the inclusion bodies, causes cell death (Ciechanover, 2001).

## 12. Animal models of PD using neurotoxins

Neurological disorders in humans can be modeled in animals using standardized procedures that recreate specific pathogenic events and their behavioral outcomes. In addition to providing an indispensable tool for basic research, animal models of human disorders allow to investigate therapeutic strategies as a prerequisite to their testing in patients (Cenci et al., 2002).

Some genetic models using transgenic mice are available, however, the use of specific neurotoxins reproducing specific features of PD, is more common. Thus, agents that selectively disrupt or destroy catecholaminergic systems, such as 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) have been used to develop PD models (Kahle et al., 2002). Recently, it has been reported that agricultural chemicals, such as rotenone and paraquat, when administered systemically, can induce specific features of PD (Betarbet et al., 2002). A common feature of these substances is that they all act on mitochondria, either by inhibiting mitochondrial complex I or complex III (Fig. 6).

### 12.1. 6-OHDA

6-Hydroxy-dopamine (6-OHDA) was the first chemical substance discovered that shows a specific neurotoxic effects on central monoaminergic neurons (Ungerstedt, 1968). 6-OHDA uses the same catecholaminergic transport system



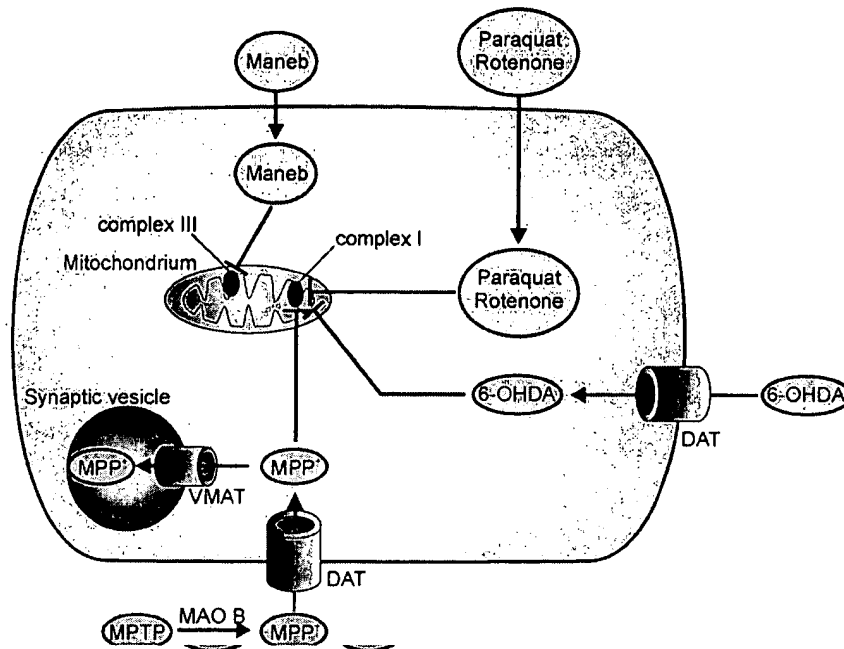


Fig. 6. The neurotoxins used in animal models of PD induce mitochondrial dysfunction. MPTP is converted by monoamine oxidase B (MAO B) to MPP<sup>+</sup>. Like 6-OHDA, MPP<sup>+</sup> is taken up by dopamine transporters and then can be accumulated by mitochondria, leading to complex I inhibition. In contrast to 6-OHDA, MPP<sup>+</sup> can be taken up by vesicular monoamine transporters, which reduces the toxicity of MPP<sup>+</sup>. Paraquat and rotenone also inhibit mitochondrial complex I, whereas maneb inhibits the mitochondrial complex III.

as norepinephrine and DA and produces specific degeneration of catecholaminergic neurons (Betarbet et al., 2002). 6-OHDA seems to be toxic to the mitochondrial complex I and induces the generation of reactive oxygen species (ROS) (Betarbet et al., 2002).

Since 6-OHDA is not able to cross the blood–brain barrier, it has to be applied directly into the substantia nigra or the striatum. Nigral 6-OHDA injection causes neuron death in the SNpc as early as 12 h post-injection, followed by striatal fiber degeneration (Jeon et al., 1995). Intrastriatal injections of 6-OHDA lead to a retrograde-induced cell death in the SNpc (Berger et al., 1991) and an approximately 70% loss of striatal DA (Przedborski et al., 1995). The magnitude of the lesion depends on the amount of 6-OHDA injected, the site of injection and the species used (Betarbet et al., 2002). At least in mice, rats, cats and primates, 6-OHDA is an effective toxin for DAergic neurons (Beal, 2001).

Animals with bilateral 6-OHDA lesions show essential elements of Parkinsonian motor symptoms, however, the bilateral lesions are not the common model, as they require intensive nursing care (Cenci et al., 2002). Thus, 6-OHDA is usually injected unilaterally, while the intact hemisphere serves as internal control. This unilateral 6-OHDA injection represents the so-called “hemiparkinson model” (Perese et al., 1989), which is characterized by an asymmetric motor-circling behavior after administration of DAergic drugs, due to the physiological imbalance between the lesioned and unlesioned striatum (Betarbet et al., 2002). Therefore, animals will rotate contralaterally to the hemisphere in which

dopamine receptor stimulation is stronger. This means that they will turn towards the side of the lesion after challenge with dopamine-releasing drugs and away from it after treatment with L-DOPA or dopamine agonists (Ungerstedt, 1976). Rotation behavior can be quantified and correlated with the degree of lesion, a major advantage of this model of PD (Beal, 2001).

The 6-OHDA model, however, does not mimic all pathological or clinical features of PD. Reports on Parkinsonian-like tremor are rare in studies of 6-OHDA-lesioned rodents; however, occasional akinesia, rigidity and tremor have been observed (Lindner et al., 1999; Cenci et al., 2002). 6-OHDA does not affect other brain areas, known to be involved in PD, such as the locus coeruleus, nor does 6-OHDA treatment induce the formation of cytoplasmatic inclusion bodies. Furthermore, this model exclusively induces acute effects, which is different from the slow progressive nature of human PD (Betarbet et al., 2002).

## 12.2. MPTP

One of the most compelling evidences for an environmental factor in PD relates to the toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). MPTP is a bypass product of the chemical synthesis of a meperidine analog with potent heroin-like properties. Drug addicts who took MPTP accidentally developed a syndrome that clinically and pathologically resembled PD (Langston et al., 1983).

MPTP is highly lipophilic and readily crosses the blood–brain barrier. MPTP mediated toxicity is induced through conversion to the 1-methyl-4-phenyl-2,3-dihydropyridium ion (MPP<sup>+</sup>) in astrocytes by monoamine oxidase B (Nicklas et al., 1985). To exert its toxicity MPP<sup>+</sup> must be transported into DAergic neurons by neurotransmitter transporters and once inside the neuron, it is thought that MPP<sup>+</sup> acts by inhibiting the electron-transport system of the mitochondrial complex I, resulting in cellular energy failure (Nicklas et al., 1987) and the formation of superoxide anions (Dawson, 2000). The selective toxicity of MPP<sup>+</sup> for DAergic neurons derives, at least in part, from its high affinity to the dopamine transporter DAT (Javitch and Snyder, 1984). Consequently, mice lacking this transporter are protected from MPTP toxicity (Bezard et al., 1999). MPP<sup>+</sup> is also sequestered into synaptic vesicles by the vesicular monoamine transporter (VMAT), and sequestration into vesicles decreases MPP<sup>+</sup> toxicity by preventing its interaction with mitochondria (Reinhard et al., 1987; Liu et al., 1992). Thus, mice with a 50% depletion of VMAT2 show increased vulnerability to MPTP (Takahashi et al., 1997).

The treatment of primates or rodents with the neurotoxin MPTP represents an animal model, which reflects many features of human PD (Speciale, 2002). In primates, depending on the application paradigm used, MPTP can produce an irreversible and severe Parkinsonian syndrome that replicates nearly all features of PD, including rigidity, tremor, slowness of movement and even freezing (Przedborski et al., 2001).

In mice, the systemical or intracranial application of MPTP can lead to severe damage of the nigrostriatal DAergic system (Fig. 7), including symptoms of motor control disturbances, resembling those in human PD, such as akinesia, rigidity, tremor, gait and posture abnormalities (Sedelis et al., 2001). In MPTP-injected mice, a dramatic loss of DAergic neurons has been detected. This cell loss is accompanied by an increase in the number of  $\alpha$ -synuclein-immunoreactive neurons located in the SNpc (Vila et al., 2000) and in an increase of  $\alpha$ -synuclein mRNA (Kuhn et al., 2003).

However, in the cases when MPTP-treated animals develop  $\alpha$ -synuclein-inclusion bodies, these aggregates usually failed to reproduce the structure of LBs (Maries et al., 2003). For example, MPTP-treated baboons develop fine granular  $\alpha$ -synuclein accumulations and some larger deposits, but these inclusions do not seem to represent full developed LBs (Kowall et al., 2000). These synuclein aggregates might be indicative for an early time-point of LB formation according to the stages proposed by Wakabayashi et al., 1998.

Regimen of MPTP administration in mice have been shown to determine the mode of neuronal cell death in the substantia nigra. Chronic regimen causes apoptotic cell death of DAergic neurons (Tatton and Kish, 1997), whereas an acute regimen of MPTP causes necrotic cell death of DAergic neurons in the substantia nigra (Jackson-Lewis et al., 1995). In addition, acute regimen of MPTP does not only result in death of DAergic neurons but also, at least

during the first four days post-injection, in a loss of the DAergic phenotype without necessarily destroying the neuron (Jackson-Lewis et al., 1995). It should be mentioned that mouse strains differ in their sensitivity to MPTP. Especially the C57/B16 strain was found to be more sensitive to systemic injection of MPTP and highly selective in terms of targeting the nigrostriatal DAergic neurons than other mouse strains (Schmidt and Feger, 2001).

In contrast to mice, rats behave relatively insensitive to MPTP (Giovanni et al., 1994b). Thus, rats injected with doses of MPTP (mg/kg) comparable to those in mice do not exhibit any significant DAergic neurodegeneration. Exclusively, the injection of high MPTP doses causes DAergic neurodegeneration in rats. However, these rats have to be pretreated with guanethidine to prevent peripheral catecholamine release and extensive mortality (Giovanni et al., 1994a).

Most of the current evidences on the mechanisms of cell death in PD originate from studies using the MPTP model (Dunnett and Bjorklund, 1999), indicating that radical scavengers, iron chelators, dopamine agonists, nitric oxide synthase inhibitors and certain calcium channel antagonists provide neuroprotection if administered prior to the intoxication (Grunblatt et al., 2000). However there are some limitations of the MPTP model. In many cases acute MPTP treatments were performed, which do not mimic the progressive degeneration of nigrostriatal DAergic neurons in PD. This can be overcome by a model of chronic MPTP regimens, however, long-term treatment with low doses of MPTP has resulted in recovery of motor deficits once the treatment is stopped (Betarbet et al., 2002).

### 12.3. Environmental toxins

The possibility that pesticides and other environmental toxins are involved in the pathogenesis has been suggested by several epidemiologic studies (Seidler et al., 1996; Gorell et al., 1998). For example, patients with certain polymorphisms in the enzyme glutathione transferase and exposure to pesticides revealed an increased incidence of PD (Menegon et al., 1998). Beside maneb, paraquat and rotenone, other complex I inhibitors might also act as environmental toxins (Kotake and Ohta, 2003).

The chloral-derived beta-carboline derivative 1-trichloromethyl-1,2,3,4-tetrahydro-beta-carboline (TaClo) also induces inhibition of the complex I of the mitochondrial respiratory chain (Janetzky et al., 1995) and has been discussed as a potential natural inducer of Parkinsonian-like symptoms (Bringmann et al., 1995). Biochemical lesions of the nigrostriatal system by TaClo induce a reduction of dopamine activity in the ipsilateral striatum. However, it remains to be determined whether TaClo also induces loss of DAergic neurons in the SNpc and/or leads to the formation of inclusion bodies. Furthermore, the specificity of TaClo for the DAergic system has to be determined, since it also

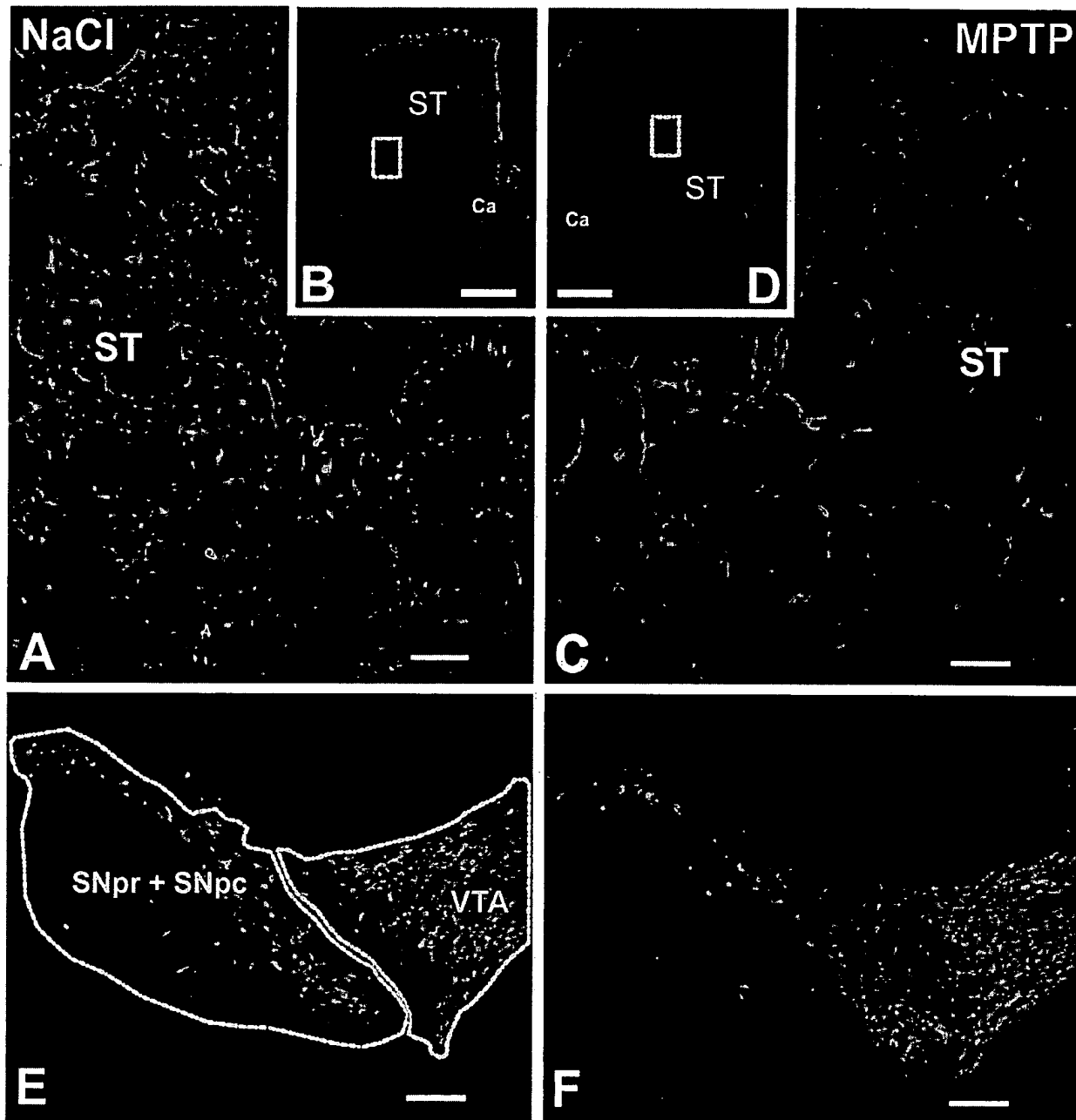


Fig. 7. MPTP treatment causes distinct changes in the nigrostriatal system. MPTP induces a significant reduction in density of striatal tyrosine hydroxylase (TH) immunoreactive (ir) dopaminergic fibers (C, D) as compared to NaCl-treated control mice (A, B). Ca: commissura anterior; ST: striatum; scale bars: (A, C) 20  $\mu$ m; (B, D) 500  $\mu$ m. In the substantia nigra—ventral tegmental area complex (E, F), the number of TH-ir dopaminergic neurons is reduced following MPTP-lesion (F) as compared to control mice (E). SNpc: substantia nigra pars compacta; SNpr: substantia nigra pars reticularis; VTA: ventral tegmental area; scale bars: (C, F) 500  $\mu$ m.

has been demonstrated that TaClo affects serotonergic cells (Bringmann et al., 2000).

A naturally occurring inhibitor of the mitochondrial complex I is annonacin. Annonacin, the major acetogenin of the tropical plant *Annona muricata* is suspected to be the cause of an atypical form of PD in Guadeloupe (Caparros-Lefebvre and Elbaz, 1999; Lannuzel et al., 2003). In mesencephalic

cell cultures it recently has been demonstrated that annonacin promotes DAergic neuronal death by impairment of energy production (Lannuzel et al., 2003). Moreover, systemically administered annonacin induces in rats a significant loss of DAergic neurons in the substantia nigra and lesions, which were similar to those in patients with atypical parkinsonism (Champy et al., 2004).

### 12.3.1. Maneb

Manganese ethylene-bis-dithiocarbamate (Mn-EBDC) is the major active element of the fungicide maneb. Maneb is an agricultural used chemical, which is known to damage the DAergic system. Exposure to the fungicide maneb could induce neurologic alterations in humans, such as postural tremor, cerebellar signs, and bradykinesia (Ferraz et al., 1988). Chronic exposure to the fungicide maneb even leads to permanent parkinsonism (Meco et al., 1994).

In the mouse, co-administration of maneb and MPTP leads to a potentiation of MPTP effects (Takahashi et al., 1989). This suggests that the exposure to a mixture of chemicals may also be relevant for the etiology of PD. Indeed, the exposure to both, paraquat and maneb, produces potentiating effects on the DAergic system (e.g. reduction of TH-ir in the striatum) compared to a treatment with one of these substances (Thiruchelvam et al., 2000, 2002).

As mentioned above, rats are relatively resistant to MPTP. However, injection of Mn-EBDC directly into the lateral ventricles of rats induces selective DAergic neurodegeneration (Zhang et al., 2003). Using this model, it has been demonstrated that Mn-EBDC-induced extensive striatal DA efflux and that Mn-EBDC preferentially inhibited mitochondrial complex III (Zhang et al., 2003).

### 12.3.2. Paraquat

Long-time exposure to paraquat (1,1'-dimethyl-4,4'-bipyridinium) has been identified as a further risk factor for PD (Liou et al., 1997). Paraquat as well as MPTP or rotenone inhibit complex I of the electron-transport-chain (Fukushima et al., 1995). Paraquat is metabolized to the paraquat radical via complex I in mitochondria and accelerates lipid peroxidation (Fukushima et al., 1995).

In animals, paraquat causes neurotoxic effects after intracerebroventricular or intracerebral injection (Corasaniti et al., 1998). Since paraquat penetrates the blood-brain barrier, it can also be applied systemically. The administration of paraquat in mice produces losses of DAergic neuron in the substantia nigra (Brooks et al., 1999; McCormack et al., 2002) and reduction in the density of striatal TH-ir fibers (Brooks et al., 1999). Constant exposure to low levels of paraquat could lead to vulnerability of DAergic neurons in the nigrostriatal system and seems to potentiate neurodegeneration caused by the exposure to other substances (like MPP<sup>+</sup>) and aging (Shimizu et al., 2003). Moreover, exposure to paraquat increases levels of  $\alpha$ -synuclein in the brain, which is accompanied by aggregate formation (Manning-Bog et al., 2002). However, cell losses were not restricted to the substantia nigra; one study reports that systemic administration of paraquat in rats also leads to cell losses in the piriform cortex (Corasaniti et al., 1998).

### 12.3.3. Rotenone

Rotenone is a naturally occurring toxin and a commonly used pesticide. Rotenone represents a mitochondrial toxin that selectively inhibits the mitochondrial complex I at the

same site as MPP<sup>+</sup>. In contrast to MPP<sup>+</sup>, rotenone is highly lipophilic and does not depend on dopamine transporter for cellular entry and furthermore, is not sequestered into synaptic terminals (Dauer et al., 2002). In 1985, it was reported that stereotaxic administration of both MPP<sup>+</sup> and rotenone caused damage to the DAergic nigrostriatal pathway in animals (Heikkila et al., 1985). Later it was found that infusion of rotenone alone also induce those effects (Greenamyre et al., 1999).

Inhibition of complex I by the lipophilic pesticide rotenone results in highly selective nigrostriatal DAergic degeneration (involving caspase-3-mediated apoptosis (Ahmadi et al., 2003)) and  $\alpha$ -synuclein-positive cytoplasmic aggregates in nigral neurons (Uversky et al., 2001; Sherer et al., 2003).

Furthermore, an increased striatal DA turn-over (Thiffault et al., 2000) and reduced TH-ir in the caudate putamen of rodents were observed (Alam and Schmidt, 2002). These alterations were associated behaviorally with hypokinesia and rigidity (Betarbet et al., 2000). Since rotenone is extremely hydrophobic and insoluble in aqueous solvents, it has to be applied by intravenous infusion through the jugular vein using subcutaneous osmotic minipumps (Betarbet et al., 2000).

However, there is also a debate concerning the specificity of rotenone for the DAergic system, since it also has been demonstrated that systemic administration of rotenone produces selective damage in the striatum but not in the substantia nigra (Ferrante et al., 1997). Moreover, infusion of rotenone does not only induce loss of striatal DAergic fibers but also of other types of striatal fibers, like serotonergic fibers (Hoglinger et al., 2003). This raises the questions whether rotenone exclusively acts on mesencephalic dopaminergic neurons, or whether other striatal projection systems are similarly affected. This issue, with regard to the DAergic specificity of rotenone, has to be clarified soon.

## 13. Conclusions and outlook

Although the available pieces of the puzzle of PD may not be complete nor assembled in the correct order as yet, the role of abnormally folded proteins in the production of the pathology merits further investigations. The emerging pattern suggests that misfolded proteins or disturbances in systems, which are involved in the clearance of misfolded proteins, may contribute to PD. Animal models, using neurotoxins to induce PD-like phenotypes, or the use of genetically manipulated animals will help to get more insight in the mechanisms and the interactions of proteins and enzymes, which result, on the one hand, in the destruction of specific neuron populations in the brain and on the other hand, in the formation of cytoplasmatic  $\alpha$ -synuclein-inclusion bodies. Currently, none of the genetic models, with deletion or target overexpression of  $\alpha$ -synuclein, and none of the models using neurotoxins fully recapitulate all key features, which clinically and pathologically characterize PD. However, each

of these available animal models recapitulates specific features of PD. Combinatorial studies using multiple models may provide a better insight into the pathogenesis of PD. However, a great breakthrough would be the development of a model which shows a slow progressive development of the main characteristic features of PD, namely the slow and progressive formation of  $\alpha$ -synuclein-inclusion bodies, which in their developmental time-course resemble the Braak-stages. Furthermore, it should be kept in mind, that neuronal destruction is not only limited to the substantia nigra, but also involves other brain areas.

Ultimately, the ideal model would feature all characteristics of PD, but one has to take into account that new insights and unforeseen pathogenic mechanisms might be discovered in the future. However, even with the models available, great advances have been made and will be made in the understanding of mechanisms involved in PD.

To date, several fundamental questions remain to be elucidated:

- Where do the first inclusion bodies occur or is there a Braak's stage zero?
- What enables the spread of the disease from lower brain areas to the forebrain?
- What triggers the misfolding of  $\alpha$ -synuclein?
- How do neurons control  $\alpha$ -synuclein stability and conformation?
- What triggers the formation of the cytoplasmatic inclusion bodies?
- What is the relationship between misfolded  $\alpha$ -synuclein and cell death?
- Are the LBs beneficial for DAergic neurons?
- What are the functions of  $\alpha$ -synuclein and synphilin-1 in non-diseased brains?

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# Neuroprotection through Delivery of Glial Cell Line-Derived Neurotrophic Factor by Neural Stem Cells in a Mouse Model of Parkinson's Disease

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Neural stem cells (NSCs) have been proposed as tools for treating neurodegeneration because of their capacity to give rise to cell types appropriate to the structure in which they are grafted. In the present work, we explore the ability of NSCs to stably express transgenes and locally deliver soluble molecules with neuroprotective activity, such as glial cell line-derived neurotrophic factor (GDNF). NSCs engineered to release GDNF engrafted well in the host striatum, integrated and gave rise to neurons, astrocytes, and oligodendrocytes, and maintained stable high levels of GDNF expression for at least 4 months. The therapeutic potential of intrastriatal GDNF-NSCs grafts

was tested in a mouse 6-hydroxydopamine model of Parkinson's disease. We found that GDNF-NSCs prevented the degeneration of dopaminergic neurons in the substantia nigra and reduced behavioral impairment in these animals. Thus, our results demonstrate that NSCs efficiently express therapeutic levels of GDNF *in vivo*, suggesting a use for NSCs engineered to release neuroprotective molecules in the treatment of neurodegenerative disorders, including Parkinson's disease.

**Key words:** dopaminergic neurons; glial cell line-derived neurotrophic factor; GDNF; neuroregeneration; neurotrophic factors; striatum; transplantation

Neurodegenerative disorders are characterized by a progressive and specific loss of neurons. In human Parkinson's disease (PD), the second most common neurodegenerative disorder, clinical symptoms appear after 50–60% neuronal loss has occurred in the substantia nigra (McGeer et al., 1988). It is this decrease in neuronal number, and the associated massive (80%) depletion of striatal dopamine levels (Bernheimer et al., 1973), that produces the characteristic tremor, rigidity, and hypokinesia of the disorder (Carlsson, 1993; Hornykiewicz, 1993). Current treatment strategies for PD focus on restoring the depletion of dopamine, generally through the administration of the dopamine precursor L-DOPA. However, because this treatment does not address the cause of the disorder or the progressive death of dopaminergic neurons, such therapy is destined to provide only temporary relief of symptoms (Olanow and Tatton, 1999).

In recent years, several therapeutic strategies have been proposed that directly address cell loss in neurodegenerative diseases

(Dunnett and Björklund, 1999). Two of the most promising are the direct replacement of dead or damaged neurons via transplantation of dopamine neurons and the prevention of neuronal death with neurotrophic molecules. Intrastriatal (caudate putamen) grafting of embryonic mesencephalic tissue has been found to efficiently restore dopaminergic function in PD patients (Olanow et al., 1996; Kordower et al., 1998; Lindvall, 1999; Piccini et al., 1999). More recently, embryonic mesencephalic progenitors (Ling et al., 1998; Studer et al., 1998, 2000), neural stem cells (NSCs) (Carpenter et al., 1999; Daadi and Weiss, 1999; Ostenfeld et al., 2000), engineered NSCs to differentiate in a coordinated manner into dopaminergic neurons (Wagner et al., 1999) and embryonic stem cells (Kawasaki et al., 2000; Lee et al., 2000), have been proposed as therapeutic tools in dopamine cell replacement for PD. However, these strategies still face difficulties regarding their large-scale implementation, in part because of the poor survival of dopamine cells (Björklund and Lindvall, 2000). Thus, both cell replacement and neuroprotective strategies for PD could benefit from progress in the application of neuroprotective molecules to enhance the survival of grafted and/or endogenous dopaminergic neurons.

Strategies using neurotrophic molecules focus on preventing the progressive loss of neurons, maintaining neuronal connections and function (neuroprotection), and inducing additional regenerative responses in neurons such as increased neurotransmitter turnover and/or axonal sprouting (neuroregeneration). Up to date, several therapeutic strategies to deliver neurotrophic factors in animal models of Parkinson's disease have been explored. These include the infusion of protein (Beck et al., 1995; Boewencamp et al., 1995; Kearns and Gash, 1995; Sauer et al., 1995; Tomac et al., 1995; Gash et al., 1996), the implantation of polymer encapsulated cells (Lindner et al., 1995), the injection of viruses (Choi-Lundberg et al., 1997; Mandel et al., 1997, 1999;

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Bohn et al., 1999; Bensadoun et al., 2000; Kirik et al., 2000; Kordower et al., 2000), and the grafting of engineered neural stem or progenitor cells (Martínez-Serrano and Björklund, 1997). However, the delivery of glial cell line-derived neurotrophic factor (GDNF), a potent neurotrophic factor for substantia nigra dopaminergic neurons (Lin et al., 1993; Beck et al., 1995; Tomac et al., 1995; Åkerud et al., 1999), by neural stem cells has not yet been tested as a candidate therapeutic approach to Parkinson's disease.

In the present study, we examine whether NSCs, for their proliferative potential *in vitro* and their capacity to give rise to regionally specific cell types that integrate in the tissue in which they are grafted *in vivo* (Snyder et al., 1992; Snyder and Macklis, 1996; Weiss et al., 1996; Martínez-Serrano and Björklund, 1997; McKay, 1997; Gage, 2000), could constitute an appropriate tool to deliver neurotrophic factors in a mice model of PD. Thus, our study exploits the known activity of neurotrophic molecules and the long-term integration ability of transplanted NSCs to develop a local and stable delivery system of neurotrophic factors for PD. Our approach has been to engineer a stable clone of NSCs (c17.2 cells; Snyder et al., 1992) to release GDNF. Our results show that NSCs expressing GDNF are able to engraft in the lesioned striatum, give rise to neurons, astrocytes, and oligodendrocytes, deliver GDNF for at least 4 months, and prevent the loss of dopaminergic neurons and the behavioral impairment of mice in a model of PD.

## MATERIALS AND METHODS

**Cell culture.** c17.2 NSCs and its derivatives were grown in DMEM supplemented with 10% fetal calf serum, 5% horse serum, 2 mM glutamine, and 20  $\mu$ g/ml gentamicin (all from Life Technologies, Grand Island, NY) on uncoated 10 cm culture dishes (Falcon, Franklin Lakes, NJ) and passaged as described previously (Snyder et al., 1992).

**Construction of the GDNF-c17.2 cell line.** An IRES-bleomycin resistance gene *SacI* fragment from pIRESbleo (Clontech, Palo Alto, CA) was cloned into the blunt *EcoRI* site of the pCAGGS expression vector (Niwa et al., 1991). Then a rat GDNF cDNA was cloned into the *EcoRV* site of the pCAGGS-IRES-bleo expression vector and used for transfection. c17.2 cells were transfected with the pCAGGS-GDNF-IRES-bleo (pCAGGS-GIB) expression vector or the mock control vector, pCAGGS-IRES-bleo (pCAGGS-IB), using the calcium phosphate–glycerol technique. Transfection, selection, isolation, and amplification of the GDNF-c17.2 or the mock-transfected MT-c17.2 clones were performed as described previously (Arenas et al., 1995). In brief, the Ca-phosphate-DNA precipitate was added to the cells for 8 hr before glycerol shock. Selection with bleomycin started 36 hr after the glycerol shock. Two weeks later, single colonies were picked, propagated, and characterized for mRNA and protein expression, as described in the following sections. c17.2 derivatives were characterized in the undifferentiated state (in the culture media mentioned above) and, after differentiation in N2 medium [consisting of a 1:1 mixture of F12 and DMEM containing 10 ng/ml insulin, 100  $\mu$ g/ml transferrin, 100  $\mu$ M putrescine, 20 nM progesterone, 30 nM selenium, 6 mg/ml glucose, and 1 mg/ml bovine serum albumin (BSA)], in poly-D-lysine (Sigma, St. Louis, MO)-coated 10 cm culture dishes.

**GDNF ribonuclease protection assay.** Assays were performed using the RPA II Ribonuclease Protection Assay kit (Ambion, Austin, TX), following the recommendations of the manufacturer. A 368 bp antisense GDNF cRNA probe (Trupp et al., 1995) was hybridized with 10  $\mu$ g of total RNA extracted from c17.2 cells proliferating, differentiating for 1 week *in vitro*, or from the striatum, 15 d after grafting. Protected cRNA fragments were separated on a polyacrylamide gel as described previously (Trupp et al., 1995). The intensity of the labeling was quantified with a phosphorimager MD Storm 840 (Molecular Dynamics, Sunnyvale, CA), and GDNF was standardized to the content of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in every sample, as described previously (Trupp et al., 1995).

**GDNF ELISA.** The production of GDNF protein was analyzed in c17.2 and GDNF-c17.2 cell lines grown in N2 medium for 12 hr. Con-

ditioned media was collected and analyzed with a GDNF ELISA kit (Promega, Madison, WI) according to the recommendations of the manufacturer. A standard curve of pure GDNF protein provided in the kit was used to quantify the production of GDNF by the cells.

**LacZ PCR.** Striata from the grafted brains were dissected out, and DNA was extracted by a deproteinization method as described previously (Laird et al., 1991) and resuspended in 100  $\mu$ l of nuclease-free H<sub>2</sub>O. Extracted DNA (0.5  $\mu$ l) was mixed with 99.5  $\mu$ l of PCR reaction mixture: 1 $\times$  PCR buffer (Promega), 4 mM MgCl<sub>2</sub>, 200  $\mu$ M dNTPs, and 1  $\mu$ l of the *Lac Z* primers. The two *Lac Z* primers used, *Lac-Z560* (5'TCCTGAGGCCGATACTGTCTGTC3') and *Lac-Z950* (TGCCGCTCATCCGCCACATATC3'), annealed to *Lac Z* (GenBank accession number L08936) and gave a PCR product of 388 bp. The amplified fragments were separated in a 2% agarose gel and visualized with ethidium bromide.

**Surgery and transplantation.** Male wild-type or nude CD-1 mice (25–35 gm; Charles River, Uppsala, Sweden) were housed and treated according to the guidelines of the European Community (86/609/EEC) and the Society for Neuroscience, and all experiments were approved by the local ethical committee. The animals were anesthetized with pentobarbital (60 mg/kg, i.p.). c17.2 and its derivatives (in the proliferative state) were washed twice with serum-free DMEM, detached with a cell lifter (Costar, Cambridge, MA), dissociated with a fire-polished Pasteur pipette, pelleted, and resuspended at a concentration of 250,000 cells/ $\mu$ l. A total of 500,000 cells were injected in four locations at the following coordinates (in millimeters) with the incisor bar at –3: anteroposterior (AP) (bregma), 0.8; lateral (L), 1.8; dorsoventral (DV) (dura), –2.55 and –2.75; AP (bregma), 0.3; L, 2.0; DV (dura), –2.55 and –2.75. Sixteen days after grafting, some of the mice were reanesthetized and injected with 4  $\mu$ g of 6-hydroxydopamine (6-OHDA) (Sigma) in the striatum, at the following coordinates (in millimeters): AP (bregma), 0.5; L, 1.9; DV, –2.65; with the incisor bar at –3.

In some experiments, the cells were prelabeled with either <sup>3</sup>H-thymidine (185 GBq/mmol, 0.25  $\mu$ Ci/ml) for 48 hr or DiI (25  $\mu$ g/ml) for 2–4 hr. Both labeling procedures resulted in 100% labeled cells, as assessed by autoradiography or fluorescence microscopy, respectively. As control for lateral transfer of labeling, labeled cells were killed by five to six cycles of freezing and thawing before grafting. Replating of the cells showed no viable cells after freezing and thawing, but when grafted, resulted in multiple labeled cells in and around the graft, suggesting that either labels were transferred to the host brain. Thus, in the present study, all c17.2 cell variants were exclusively traced by immunohistochemistry, *in situ* hybridization (ISH), and/or PCR to detect the cell-autonomous genetic markers in the grafted cells.

**Histology.** Mice were transcardially perfused with ice-cooled 4% paraformaldehyde (PFA). Brains were post-fixed for 0–2 hr, embedded in 10% sucrose for 24 hr, and frozen on dry ice-cooled isopentane. Serial cryostat sections (14  $\mu$ m thick) through the entire substantia nigra and striatum were obtained every 200  $\mu$ m.

Sections through the striatum were incubated at 4°C overnight with one of the following antibodies in dilution buffer: rabbit anti- $\beta$ -galactosidase ( $\beta$ -Gal), 1:250 (Cappel-Worthington, Durham, NC); rabbit anti-glial fibrillary acidic protein (GFAP), 1:500 (Dako, Glostrup, Denmark); mouse anti-CNase, 1:250 (Boehringer Mannheim, Mannheim, Germany); mouse anti-NeuN, 1:100 (Chemicon, Temecula, CA); mouse anti-rat 401 (for nestin), 3  $\mu$ g/ml (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA); and goat anti-GDNF, 1:20 (R & D Systems, Minneapolis, MN) in dilution buffer (PBS containing 3% BSA and 0.3% Triton X-100). After washing, sections were incubated for 1–3 hr with the appropriate secondary antibodies: a goat anti-rabbit fluorescein isothiocyanate-conjugated antibody, 1:100 (Vector Laboratories, Burlingame, CA); a donkey anti-mouse rhodamine antibody, 1:200 (Jackson ImmunoResearch, West Grove, PA); or a biotinylated rabbit anti-goat IgG, 1:500 (Vector Laboratories), which was detected by incubation with avidin fluorescein isothiocyanate, 1:500 (Vector Laboratories) for 1 hr.  $\beta$ -Galactosidase activity was detected by incubation of the tissue in 5-bromo-4-chloro-3-indolyl  $\beta$ -D-galactosidase (X-Gal) as described previously (Snyder et al., 1992).

Sections through the substantia nigra and the striatum of 6-OHDA-lesioned animals were processed for tyrosine hydroxylase (TH) immunohistochemistry using a mouse anti-TH antibody (1:1000; Incstar, Stillwater, MN) and donkey anti-mouse rhodamine antibody (1:200; Jackson ImmunoResearch). Neurons through the entire substantia nigra were counted in serial sections, every 200  $\mu$ m, in five to seven animals per experimental group. Neurons showing a clear TH-positive cytoplasm



surrounding a nonstained nucleus were counted as positive in blind determinations using a Zeiss (Oberkochen, Germany) Axioplan 2 microscope. TH immunoreactivity was also examined in the striatum.

Double immunostainings of striatal sections with  $\beta$ -Gal/GFAP or GDNF/NeuN antibodies were performed sequentially; first,  $\beta$ -Gal or GDNF were detected, and then GFAP or NeuN immunohistochemistry was performed.  $\beta$ -Gal/NeuN,  $\beta$ -Gal/CNPase, GDNF/GFAP, and GDNF/CNPase double immunohistochemistry of striatal sections and TH/GDNF double immunohistochemistry in sections through the substantia nigra were performed by simultaneous incubation of the sections with the two primary antibodies first and the two secondary antibodies afterward. The specificity of the stainings was confirmed by comparison of the double stainings with the single stained tissue and by omission of the primary antibody.

The position that oligodendrocytes derived from GDNF-c17.2 and MT-c17.2 cells occupied with respect to CNPase-positive white matter fiber bundles of the internal capsule was assessed in striatal sections. Only cells showing clear double-labeled GDNF/CNPase or  $\beta$ -Gal/CNPase somas surrounding unlabeled nuclei at 40 $\times$  magnification were included in the study. Fifty-five randomly chosen cells in fields adjacent to the graft site were analyzed in each grafted brain, in three animals per condition.

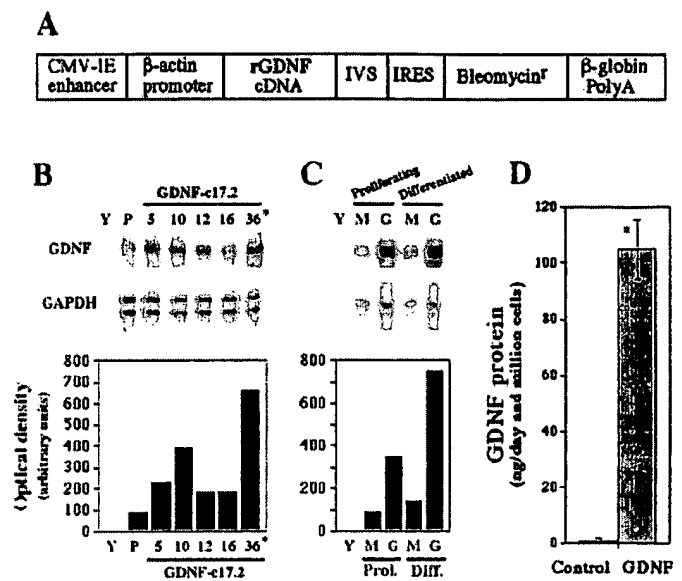
**In situ hybridization.** For ISH, either PFA-perfused or fresh frozen tissue was used. ISH with  $^{35}$ S-labeled riboprobes was performed as described previously (Trupp et al., 1997). In brief, sections were fixed for 15 min in ice-cooled 4% PFA and rinsed three times in PBS. Tissue was deproteinized in 0.2 M HCl for 10 min, acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine for 20 min, and dehydrated in increasing concentrations of ethanol. Slides were incubated 16 hr in a humidified chamber at 53°C with  $10^6$  cpm of probe in 200  $\mu$ l of hybridization cocktail. All of the washes were performed at 62°C: first, two washes of 15 min in 1 $\times$  SSC, 30 min in 50% formamide and 0.5 $\times$  SSC, and 15 min in 1 $\times$  SSC; then, 30 min RNaseA treatment (40  $\mu$ g/ml) at 37°C and two washes of 15 min in 1 $\times$  SSC before dehydration in ethanol and air drying. Slides were first exposed to  $\beta$ -Max x-ray film (Amersham Pharmacia Biotech, Buckinghamshire, UK) for 12–20 d. Subsequently, the slides were dipped in NTB-2 photoemulsion (Eastman Kodak, Rochester, NY) diluted 1:1 in water, exposed at 4°C for 6–8 weeks, developed with D19 (Eastman Kodak), fixed with AL-4 (Agfa Gevaert, Kista, Sweden), and counterstained with thionin.

**Behavioral testing.** Behavioral testing was performed 12 d after lesioning. Mice were injected subcutaneously with apomorphine (0.5 mg/kg). Ten minutes after injection, the number of rotations were scored for 5 min, an interval of time that gives a very stable response (Winkler and Weiss, 1996). One or 2 d later, the mice were assessed for amphetamine-induced turning behavior (Barneoud et al., 1995). Amphetamine (2.5 mg/kg) was injected intraperitoneally, and the number of rotations was scored for 3 min at 15, 30, and 45 min after injection. The values were expressed as net total numbers of full turns.

## RESULTS

### Engineering and characterization of GDNF expression in NSCs

c17.2 mouse NSCs were transfected with the pCAGGS-GIB expression vector (GDNF-c17.2 cells) (Fig. 1A) or the pCAGGS-IB vector (MT-c17.2 cells). c17.2, GDNF-c17.2, and MT-c17.2 clones showed similar morphology, survival, and proliferation rate in the undifferentiated state. Initially, we examined GDNF mRNA expression by ribonuclease protection assay (RPA) in undifferentiated, proliferating individual clones (Fig. 1B). To verify stability of transgene expression after differentiation *in vitro*, the highest GDNF expressor (GDNF-c17.2 cell line) and a randomly chosen mock clone (MT-c17.2 cell line) were subsequently tested for expression of GDNF after culture in serum-free medium for 1 week. RPA analysis of these cells showed persistent high levels of GDNF mRNA expression in the GDNF-c17.2 clone and low levels in the MT-c17.2 clone (Fig. 1C), suggesting that no downregulation of transgene expression was occurring *in vitro*. The increased expression of GDNF mRNA did not appear to deleteriously affect the cells, because all GDNF-c17.2 clones analyzed had similar morphologies to paren-



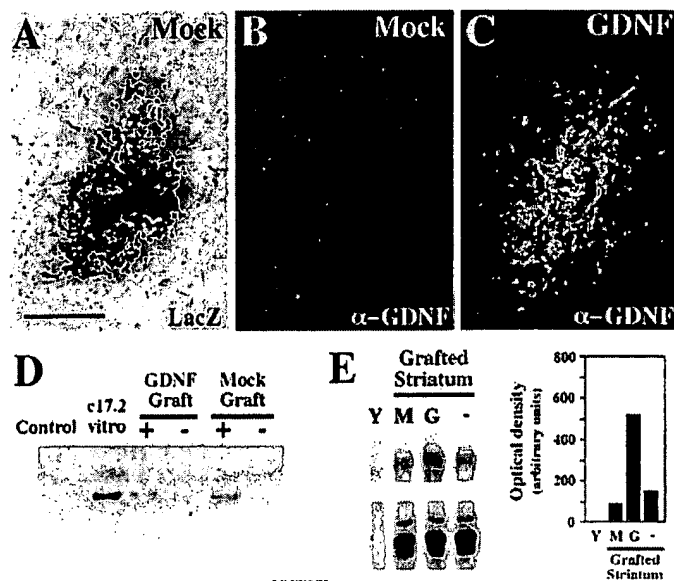
**Figure 1.** Establishment of a GDNF-overexpressing neural stem cell line. *A*, Schematic representation of the pCAGGS-GIB expression vector that was used for transfection of the c17.2 cells. A rat GDNF cDNA and a mini-intron IRES-bleo resistance gene fragment were cloned after the  $\beta$ -actin promoter and a CMV-immediate early (CMV-IE) enhancer, in the pCAGGS vector. *B* and *C* show two representative experiments, in which GDNF mRNA was analyzed by RPA in control and GDNF-transfected cell lines. *IVS*, Synthetic intron. *B*, GDNF mRNA expression was determined in the parental c17.2 cell line (*P*) and five clones (5, 10, 12, 16, 36) transfected with the pCAGGS-GIB construct. Yeast tRNA (*Y*) was used as a negative control. Clone 36, the highest expressor, was named GDNF-c17.2 and further characterized. *C*, GDNF mRNA expression was also examined in proliferating and differentiated (in N2 medium for 1 week) MT-c17.2 control cells (*M*) and the GDNF-c17.2 cells (*G*). Note that the difference in GDNF mRNA expression between the MT-c17.2 and the GDNF-c17.2 cell lines is even greater after differentiation than while proliferating. *D*, ELISA analysis of supernatants from proliferating cells *in vitro* showed that the control cells (Control; c17.2 or MT-c17.2) released <1 ng of GDNF per  $10^6$  cells in 1 d, whereas the GDNF-c17.2 cell line produced ~100 ng of GDNF per  $10^6$  cells in 1 d ( $n = 3-4$ ; \* $p < 0.0001$ ; unpaired Student's *t* test).

tal and MT-c17.2 cell lines after differentiation in N2 media, in the absence of mitogen. To test whether increased levels of mRNA resulted in increased, sustained release of GDNF protein, we quantified the amount of GDNF protein secreted in the media by ELISA. Whereas the parental c17.2 and MT-c17.2 cell lines released <1 ng of GDNF per  $10^6$  cells in 1 d, the selected GDNF-c17.2 clone released ~100 ng of GDNF per  $10^6$  cells in 1 d (Fig. 1D).

### Detection of NSCs after intrastriatal grafting *in vivo*

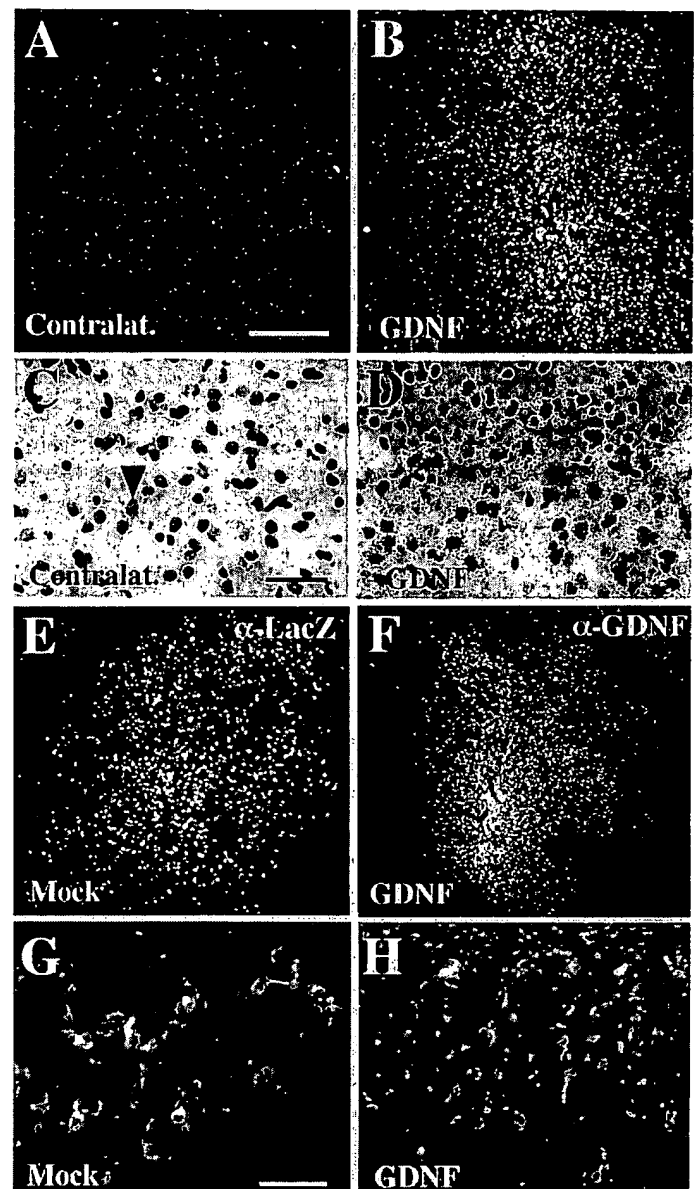
Because transplanted NSCs disperse, integrate, and assume local phenotypes among endogenous host cells, reliable tracing becomes a key issue. The parental c17.2 carries a  $\beta$ -galactosidase reporter that makes their identification by X-Gal histochemistry or anti- $\beta$ -gal immunohistochemistry possible. Whereas MT-c17.2 cells expressed  $\beta$ -galactosidase at early (Fig. 2A) and late (Fig. 3E,G) postgrafting time points, we found a downregulation of this reporter in GDNF-c17.2 cells *in vivo* and no expression was detected from 4.5 d to 4 months after intrastriatal grafting of the GDNF-NSCs (Fig. 3G). We also found that other c17.2 derivatives downregulated the *lacZ* transgene at distinct time points after differentiation, suggesting that this phenomenon may be related to the integration site of the new transgene and/or to the





**Figure 2.** GDNF-c17.2 and MT-c17.2 grafted cells survive the grafting procedure well, but only GDNF-c17.2 cells express high levels of GDNF *in vivo*. *A–C*, MT-c17.2 and GDNF-c17.2 grafted cells in the striatum 4.5 d after grafting. *A*, X-Gal histochemistry (*LacZ*) confirmed the presence of MT-c17.2 grafted cells. Note the migration out of the graft by some of the cells as soon as 4.5 d. *B*, No GDNF immunoreactivity was detected in MT-c17.2 grafts on adjacent slides. *C*, GDNF immunohistochemistry showed many GDNF-positive cells in the GDNF-c17.2 grafts, which also were found migrating away from the graft site at this time point. *D*, The presence of cells in the grafted striatum was also verified at later time points (1 and 10 months) by PCR against the *lacZ* vector. Cells could be detected in the GDNF or mock grafted striatum (+) but not in the contralateral striatum (–). The signal was similar at 1 month (as shown in the *Mock Graft*) and at 10 months (shown in the *GDNF Graft*). Proliferating c17.2 cells (*c17.2 vitro*) were used as positive control, and cells not transfected with *lacZ* expression vectors were used as negative control. *E*, Increased levels of GDNF mRNA expression could also be detected by RPA in the striatum 15 d after grafting of the GDNF-c17.2 cells (*G*) but not after grafting the MT-c17.2 cells (*M*). The levels of expression of GDNF in the non-grafted striatum (–) were higher than in the control grafted striatum (*M*) because the MT-c17.2 cells express less GDNF than the intact adult striatum. GDNF expression in the GDNF-c17.2 grafted striatum was more than five times higher than in the MT-c17.2 grafted striatum. Yeast tRNA was used as negative control. Values correspond to one representative experiment ( $n = 2$ ). Scale bar (in *A*): *A–C*, 250  $\mu$ m.

clonal expansion of the cells. However, the presence of grafted GDNF-c17.2 or MT-c17.2 cells could be determined in a reliable manner by PCR against the *lacZ* cDNA sequences, because those sequences are present in c17.2 cells (Snyder et al., 1992). As shown in Figure 2*D*, that technique allowed us to detect either GDNF-c17.2 or MT-c17.2 cells after grafting. In addition, the presence of GDNF-c17.2 cells in the brain was detected by GDNF immunohistochemistry (Fig. 2*C*) or RPA (Fig. 2*E*) in all animals grafted for up to 2 weeks. Interestingly, anti-GDNF antibodies were only able to detect the high levels of GDNF overexpressed by the GDNF-c17.2 cells (Fig. 3*F,H*) but not the basal levels of expression in MT-c17.2 cells or in the striatum (Fig. 4*A*). Moreover, because none of the MT-c17.2 grafted brains contained cells expressing GDNF mRNA or protein above control or contralateral grafted striata (Fig. 2*B*), our results indicate that grafting of the c17.2 cells does not induce GDNF expression in the host tissue. Thus, combined, our results are consistent with the notion that all cells expressing high levels of GDNF mRNA derive from the GDNF-c17.2 cells and that the

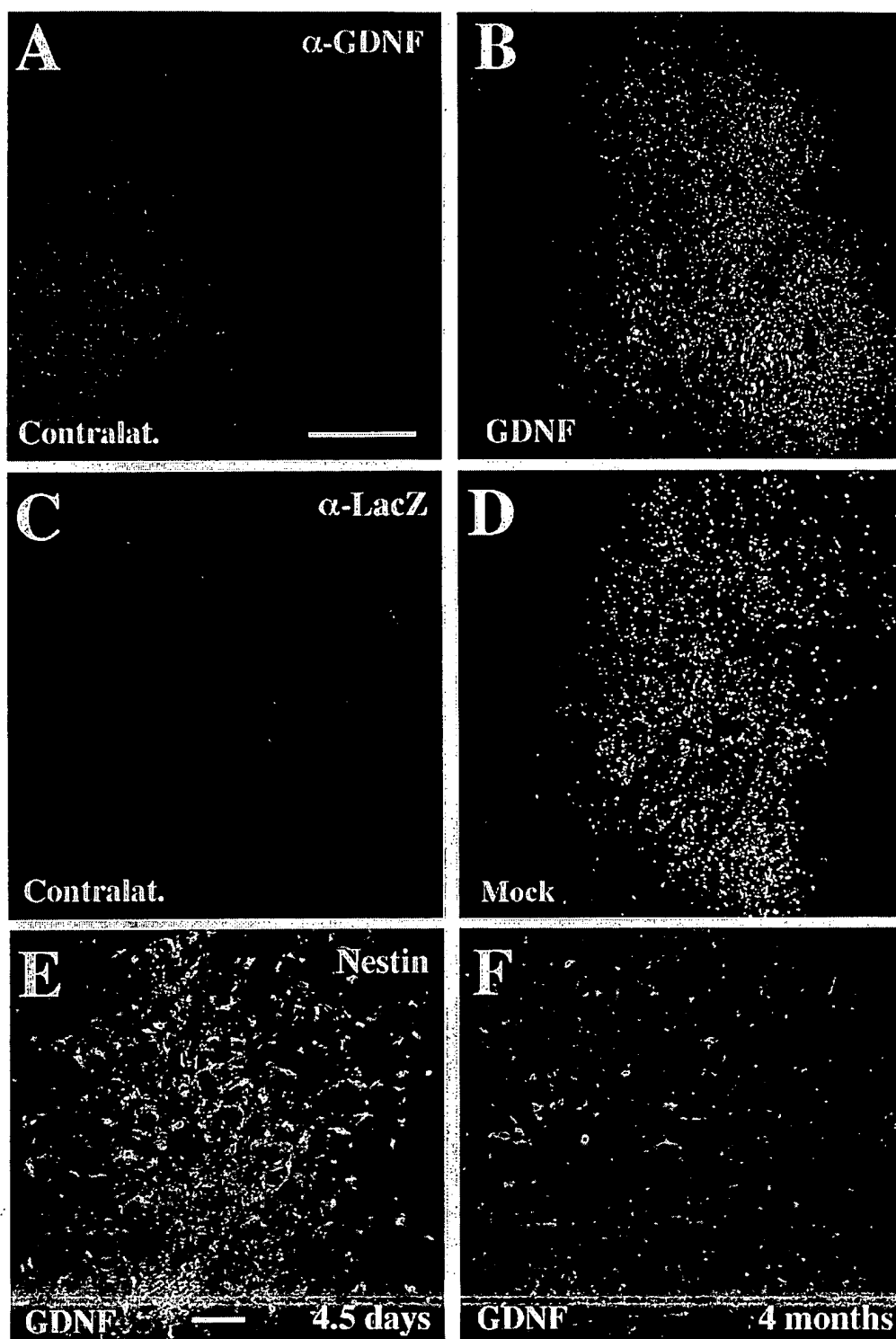


**Figure 3.** GDNF-c17.2 and MT-c17.2 cells engraft well and disperse within the striatum by 1 month after grafting. *A–D*, ISH showed the presence of cells expressing very high levels of GDNF mRNA in the GDNF grafted striatum (*B, D*) but not in the contralateral side (*A, C*). Note the abundance and dispersion of the signal in the grafted striatum (*B*). At higher magnification and bright field (*C, D*), it is possible to observe one endogenous GDNF-expressing cell (arrowhead in *C*) and many grafted cells with high levels of GDNF mRNA expression (*D*). *E, G*, The presence of MT-c17.2 cells was verified by  $\beta$ -Gal immunohistochemistry against the *lacZ* product ( $\alpha$ -Lac) at 30 d after grafting.  $\beta$ -Gal-immunoreactive cells (magnified in *G*) were detected in the ipsilateral striatum to the graft (*E*). *F, H*, GDNF-c17.2 cells were also detected 1 month after grafting by GDNF immunohistochemistry. *F*, GDNF-c17.2 cells (magnified in *H*) displayed a similar distribution to cells expressing high levels of GDNF mRNA. Scale bars: (in *A*) *A, B, E, F*, 500  $\mu$ m; (in *C*) *C, D*, 100  $\mu$ m; (in *G*) *G, H*, 100  $\mu$ m.

GDNF transgene is expressed for at least 2 weeks after grafting at sustained high levels in all animals.

#### Graft survival after intrastriatal grafting *in vivo*

We next examined whether GDNF-c17.2 and MT-c17.2 cells, which have a mixed CD-1 and C57BL/6 genetic background (Snyder et al., 1992), survived for up to 4 months after grafting in



**Figure 4.** GDNF-c17.2 and MT-c17.2 engrafted for at least 4 months, the longest time point analyzed morphologically. *A, B*, GDNF-c17.2 cells engrafted well in the adult striatum and were detected by GDNF immunohistochemistry in the ipsilateral striatum of all nude mice but not in the contralateral striatum (*A*). MT-c17.2 cells showed a similar pattern of engraftment and were readily detected by  $\beta$ -Gal immunohistochemistry against the *lacZ* product ( $\alpha$ -Lac) 4 months after grafting in the ipsilateral striatum (*D*) but not in the contralateral side (*C*). Scale bar (in *A*): *A–D*, 500  $\mu$ m. *E, F*, At 4 months after grafting, we also found that GDNF-c17.2 cells differentiated and did not express the neural stem cell marker nestin (*F*). Instead, nestin was found to be abundantly expressed by most of the grafted cells by 4.5 d (*E*). Scale bar (in *E*): *E, F*, 100  $\mu$ m.

the adult striatum of CD-1 mice. The brains of animals receiving the allografts were analyzed by immunohistochemistry and/or *in situ* hybridization after 15 d, 1 month, or 4 months. Fifteen days after grafting, the grafts were present in 100% of the animals receiving either the MT-c17.2 or GDNF-c17.2 grafts, as assessed by  $\beta$ -Gal or GDNF immunohistochemistry, respectively (Table 1). One month after grafting, both cell lines were also detected by ISH (Fig. 3*B, D*) or immunohistochemistry (Fig. 3*E–H*) in the ipsilateral side. However, only 41.6% ( $n = 12$ ) of the GDNF-c17.2

grafted brains and 37.5% ( $n = 8$ ) of the MT-c17.2 grafted brains showed positive cells at this time point (Table 1). This percentage of engraftment was similar to that reported previously for c17.2 cells after 6 weeks (Snyder et al., 1997) and suggested to us that engraftment in the adult brain does not depend on clonality or transgene expression but on the time after grafting. Similarly, GDNF-c17.2 cells were identified by PCR against the *lacZ* cDNA in one animal of three at 1 month and in one animal of four at 10 months after grafting (Fig. 2*D*). In agreement with this, the

**Table 1.** Engraftment efficiency of MT-c17.2 or GDNF-c17.2 cells in the adult striatum of wild-type and nude CD-1 mice

	14 d WT	30 d WT	120 d WT	120 d Nude
MT-c17.2 grafts	3 / 3	3 / 8	0 / 8	3 / 3
( $\beta$ -Gal-positive)	(100%)	(37.5%)	(0%)	(100%)
GDNF-c17.2 grafts	4 / 4	5 / 12	1 / 8	3 / 3
(GDNF-positive)	(100%)	(41.6%)	(12.5%)	(100%)

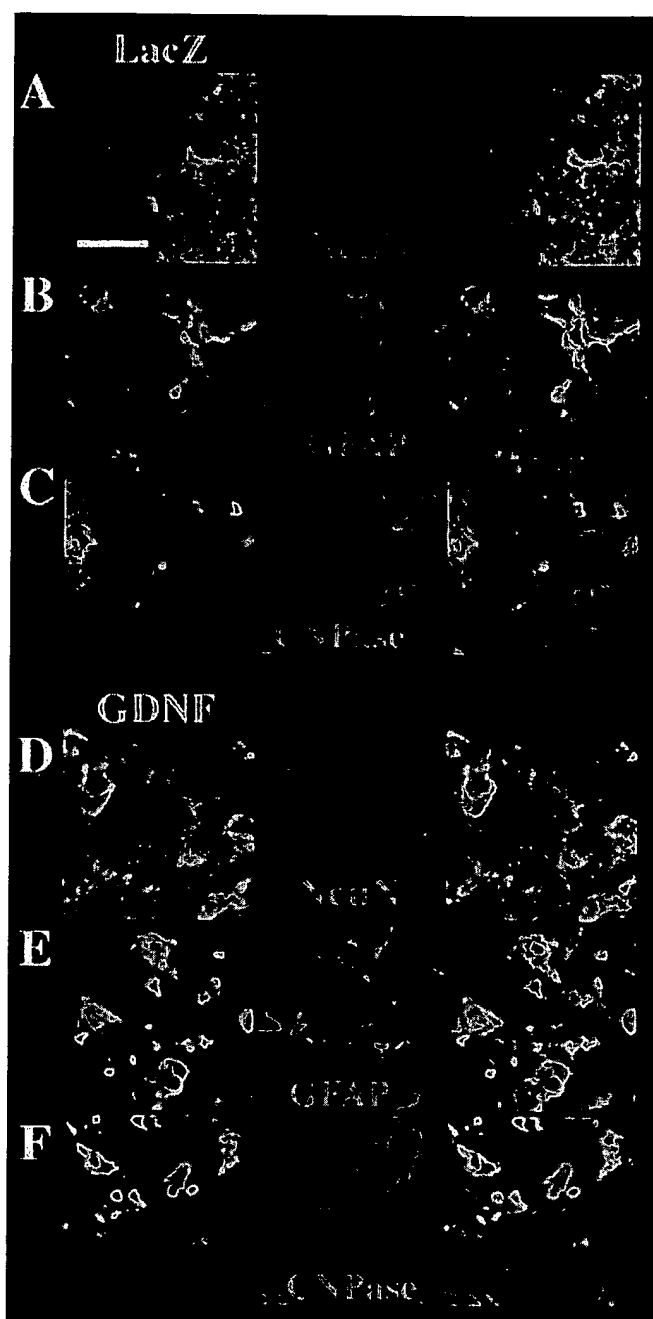
Animals showing  $\beta$ -Gal- or GDNF-immunoreactive cells in the striatum at the indicated time points were considered as positive. In general, most of the animals showed either a large engraftment (see Figs. 3, 4) or absence of positive cells. The engraftment efficiency of MT-c17.2 or GDNF-c17.2 cells was 100% in nude CD-1 mice (Nude) but declined over time in wild-type mice (WT).

percentage of animals showing engraftment at 4 months decreased even more to reach 12.5% ( $n = 8$ ) in animals receiving GDNF-c17.2 cells and to 0% ( $n = 8$ ) in animals receiving MT-c17.2 grafts (Table 1). Surprisingly, in the only animal in which cells were detected at 4 months, a full striatal engraftment was identified, suggesting that, although progressive, the cell loss process takes place as an all-or-nothing process with important individual variation. We therefore decided to examine whether the immune response of the host compromised the survival of the grafts and performed grafting experiments in CD-1 nude mice. Our results show that grafting of either GDNF-c17.2 cells (Fig. 4B) or MT-c17.2 cells (Fig. 4D) in adult nude CD-1 mice results in the engraftment of cells in 100% of the animals after 4 months (Table 1), as assessed by GDNF or  $\beta$ -Gal immunohistochemistry.

#### C17.2 NSCs disperse in the adult striatum to integrate, differentiate, and give rise to distinct cell lineages

Morphological analysis of those brains showing engraftment of either MT-c17.2 or GDNF-c17.2 NSCs at 1 month showed that MT-c17.2 and GDNF-c17.2 NSCs dispersed similarly throughout the striatum (Fig. 3B,E,F), suggesting that their ability to migrate in the brain is not impaired or enhanced by the expression of the *LacZ* or *GDNF* transgenes. Moreover, comparison of both cells at 1 and 4 months, GDNF-c17.2 (Figs. 3B, 4B) and MT-c17.2 (Figs. 3E, 4D), showed that most of the dispersion of the cells takes place within 1 month after grafting.

Because cell proliferation is enhanced by *v-myc* in c17.2 cells *in vitro*, we extensively examined whether the MT-c17.2 or GDNF-c17.2 cells formed brain tumors *in vivo*. By now, we already grafted >100 adult animals, including wild-type and nude mice, and we never found brain tumors. On the contrary, consistent with differentiation *in vivo*, GDNF-c17.2 cells downregulate nestin expression from 4.5 d to 4 months after grafting, as assessed by immunohistochemistry (Fig. 4E,F). Thus, our results indicate that MT-c17.2 and GDNF-c17.2 cells differentiate *in vivo* as much as parental c17.2 cells do (Snyder et al., 1992). We next analyzed the phenotype that differentiated GDNF-c17.2 cells adopted after grafting, using double immunohistochemistry with antibodies against GDNF or  $\beta$ -Gal and NeuN to identify GDNF-c17.2- or MT-c17.2-derived neurons, GDNF or  $\beta$ -Gal and GFAP to identify c17.2 cell-derived astrocytes, and GDNF or  $\beta$ -Gal and CNPase to identify GDNF-c17.2- or MT-c17.2-derived oligodendrocytes. One month after grafting, MT-c17.2 cells were detected as  $\beta$ -Gal/NeuN double-positive cells (Fig. 5A),  $\beta$ -Gal/GFAP double-positive cells (Fig. 5B), and  $\beta$ -Gal/CNPase double-positive cells (Fig. 5C). In contrast, GDNF-c17.2 grafts gave rise to few GDNF/NeuN double-positive neurons (Fig. 5D), few GDNF/GFAP double-positive astrocytes (Fig. 5E), and a high



**Figure 5.** Double immunohistochemistry showing that MT-c17.2 (A–C) and GDNF-c17.2 cells (D–F) behave as multipotent NSCs after intrastriatal grafting in the adult CD-1 mice and give rise to neurons (A, D), astrocytes (B, E), and oligodendrocytes (C, F). A–C, Double immunohistochemistry with anti- $\beta$ -Gal antibodies (*LacZ*, in green), and antibodies against NeuN (A), GFAP (B), and CNPase (C), all in red, showed that MT-c17.2 can give rise to all three neuronal lineages *in vivo*. D–F, Double immunohistochemistry with anti-GDNF antibodies (in green) and antibodies against NeuN (D), GFAP (E), and CNPase (F), all in red, showed that GDNF-c17.2 can give rise to all three neuronal lineages. Note that most GDNF-c17.2 cells stained with anti-CNPase antibodies, suggesting that they mainly become oligodendrocytes (see Table 2). Scale bar (in A): A–F, 25  $\mu$ m.

proportion (81%) of GDNF/CNPase double-positive oligodendrocytes in animals grafted for 1 month (Fig. 5F, Table 2). This result was confirmed in animals grafted for 4 months, indicating that GDNF-c17.2 cells adopt a stable oligodendrocytic fate.

**Table 2. GDNF-c17.2 cells gave rise to a higher proportion of CNPase-positive cells than MT-c17.2 cells and located together with endogenous CNPase-positive cells within white matter fiber bundles of the internal capsule**

	CNPase-negative cells		CNPase-positive cells	
	Gray matter	White matter	Gray matter	White matter
MT-c17.2 ( $\beta$ -Gal-positive cells)	44.5 $\pm$ 0.5 (81%)	3 $\pm$ 3 (5.5%)	3 $\pm$ 2 (5.4%)	4.5 $\pm$ 0.5 (8.1%)
GDNF-c17.2 (GDNF-positive cells)	7.7 $\pm$ 2 (13.7%)	2.3 $\pm$ 1 (5.3%)	6.3 $\pm$ 5 (11%)	39 $\pm$ 5 (70%)

Animals were grafted with either MT-c17.2 or GDNF-c17.2 cells for 1 month. The grafted cells were identified by  $\beta$ -Gal or GDNF immunohistochemistry, respectively, and assessed for double labeling with anti-CNPase antibodies. Fifty-five randomly chosen  $\beta$ -Gal- or GDNF-positive cells were analyzed per animal in three different brains per condition. Our results show that most MT-c17.2  $\beta$ -Gal-positive cells become CNPase-negative (86.4%) and are found outside of the white matter fiber bundles (81%). Instead, most GDNF-c17.2 cells became CNPase-positive (81%) and are located within white matter fiber bundles (70%).

Moreover, most GDNF-c17.2-derived cells predominantly (75.3%) integrated within the white matter and fibers bundles of the internal capsule that transverses the striatum (Fig. 5*F*, Table 2). Instead, MT-c17.2 cells gave rise to fewer CNPase-positive oligodendrocytes (13.5%), and only 13.6% of the MT-c17.2 cells were found in the white matter. Thus, our results show that GDNF-c17.2 NSCs retain the ability of the parental c17.2 cells to give rise to neurons and astrocytes, but they mainly give rise to oligodendrocytes after grafting in the adult striatum. Moreover, 70% of the GDNF-c17.2 cells gave rise to oligodendrocytes that incorporated in the adequate striatal compartment, that is, the white matter of the internal capsule.

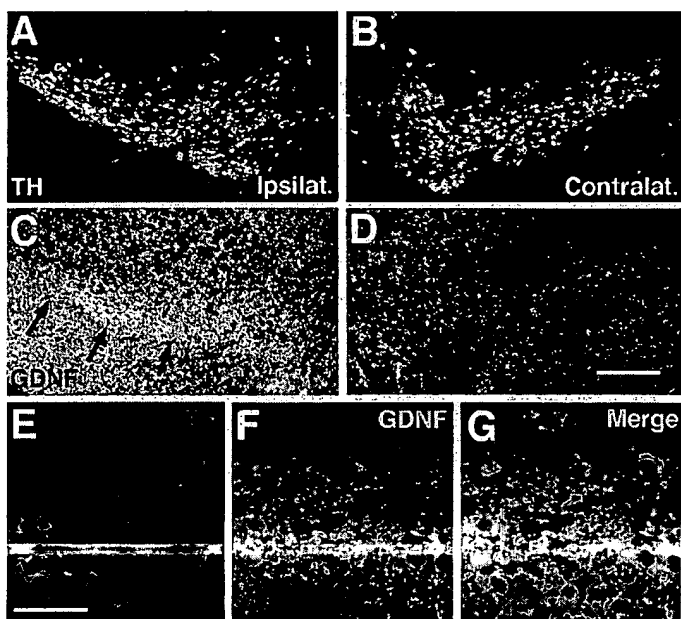
#### GDNF-expressing NSCs efficiently deliver GDNF to dopaminergic neurons

Next, we examined whether GDNF was efficiently delivered to dopaminergic neurons in the host brain. We first examined by immunohistochemistry the diffusion of GDNF in the host striatum and only found background levels of GDNF immunoreactivity in the striatal neuropil (Fig. 3*F,H*). However, when the ventral midbrain was examined, we found an increase in GDNF immunoreactivity in the ipsilateral substantia nigra to the striatal GDNF graft (Fig. 6*C*). On the contrary, in the ipsilateral substantia nigra to a striatal mock graft, or in the contralateral side to GDNF grafts (Fig. 6*D*), only background levels of GDNF immunoreactivity were detected. Moreover, double TH and GDNF immunohistochemistry clearly showed that GDNF immunoreactivity was contained both in the substantia nigra neuropil and within dopaminergic neurons (Fig. 6*E–G*), suggesting that GDNF was efficiently transported in a retrograde manner by dopaminergic neurons from the striatum to the substantia nigra.

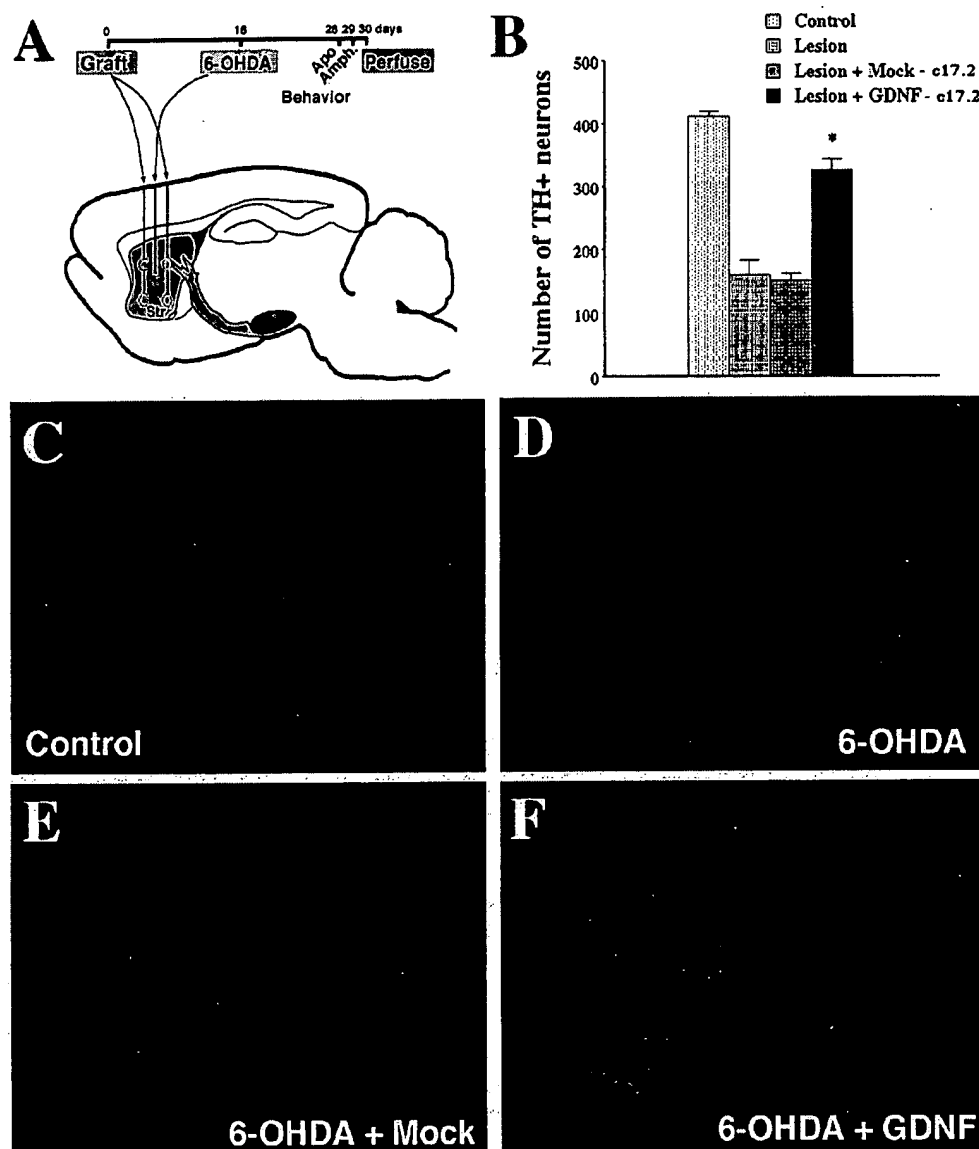
#### GDNF-expressing NSCs prevent the loss of substantia nigra dopaminergic neurons in a 6-OHDA model of PD

To test the therapeutic potential of the GDNF-c17.2 NSCs, we performed intrastriatal grafts in a 6-OHDA lesion model of PD. A total of  $5 \times 10^5$  GDNF-c17.2 or MT-c17.2 cells were grafted in four deposits in the striatum (Fig. 7*A*). Sixteen days later, grafted and nongrafted animals received a single intrastriatal injection of 4  $\mu$ g of 6-OHDA. Thirty days after grafting, we characterized the neuroprotective effect of the GDNF-c17.2 NSCs on substantia nigra dopaminergic neurons. In the group of animals injected with 6-OHDA alone or with 6-OHDA and MT-c17.2, TH immunohistochemistry demonstrated a similar loss of substantia nigra dopaminergic neurons of 61 and 63%, respectively (Fig. 7*B–E*), indicating that the NSCs had no survival-promoting effect per se.

In contrast, animals grafted with the GDNF-c17.2 NSCs demonstrated a loss of only 21% of substantia nigra dopaminergic neurons (Fig. 7*B,F*). Moreover, higher levels of TH immunoreactivity were also detected in the striatum of GDNF-c17.2 grafted animals (Fig. 8*C*) compared with 6-OHDA-lesioned or animals receiving both 6-OHDA and MT-c17.2 grafts (Fig. 8*B*). Thus, our results show that dopaminergic neurons of animals grafted with GDNF-c17.2 NSCs are more resistant to 6-OHDA and oxidative stress, a mechanism that has been invoked in the etiopathology of Parkinson's disease (Jenner and Olanow, 1998).



**Figure 6.** Intrastriatal grafting of the GDNF-c17.2 cells results in the retrograde labeling of substantia nigra dopaminergic neurons. *A–D*, Adjacent sections from the ipsilateral (*A*, *C*) or contralateral (*B*, *D*) substantia nigra to intrastriatal GDNF-c17.2 grafts were processed for immunohistochemistry with antibodies against TH (*A*, *B*) and GDNF (*C*, *D*) 1 month after grafting. GDNF immunoreactivity over background level was detected in the ipsilateral substantia nigra but not in the contralateral side. *E–G*, Double immunohistochemistry for TH (*E*) and GDNF (*F*) revealed that GDNF was contained within dopaminergic neurons in the substantia nigra pars compacta (*G*), suggesting that GDNF was retrogradely transported by substantia nigra dopaminergic neurons from the ipsilateral striatum. Scale bars: (in *D*) *A–D*, 250  $\mu$ m; (in *E*) *E–G*, 50  $\mu$ m.



**Figure 7.** GDNF-c17.2 grafts protected substantia nigra dopaminergic neurons in a 6-OHDA model of Parkinson's disease. *A*, Schematic drawing of the positions at which the cells were grafted and the 6-OHDA injection was performed. The contralateral side was left intact. The grafting was performed at day 0, the 6-OHDA injection at day 16, and perfusion at day 30. Apomorphine- and amphetamine-induced circling behavior were studied at days 28 and 29, respectively. *B*, Quantification of the number of substantia nigra TH-positive neurons in the indicated experimental conditions. Values represent the mean  $\pm$  SEM ( $n = 5-7$ ) of the number of TH-positive cells counted in serial sections through the substantia nigra. \* $p < 0.0001$  versus lesioned substantia nigra grafted or not with the MT-c17.2 cell line as determined by one-way ANOVA (significant effect of treatment,  $p < 0.0001$ ;  $F_{(3,32)} = 101.1$ ). *C-F*, TH immunohistochemistry showed that grafting of the MT-c17.2 cells did not prevent the loss of dopamine neurons (compare *D* and *E* with *C*). Instead, GDNF-c17.2 cells (*F*) prevented the loss of dopamine neurons in the substantia nigra.

#### Intrastriatal grafting of GDNF-expressing NSCs prevents behavioral abnormalities associated with unilateral 6-OHDA lesions

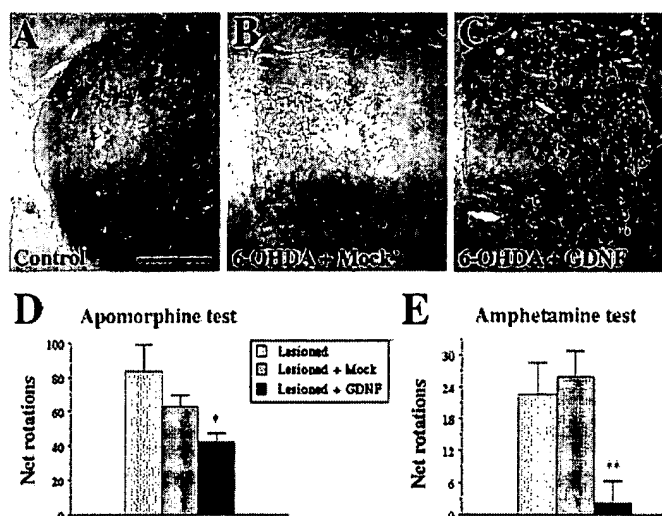
To determine whether the protective effects of the GDNF-c17.2 cells translated into functional improvement, 12 d after lesioning we assayed the grafted animals for apomorphine-induced circling behavior. Animals receiving 6-OHDA plus GDNF-c17.2 grafts showed a 50% reduction in the number of net contralateral turns compared with the group receiving only 6-OHDA, whereas animals receiving the MT-c17.2 grafts plus 6-OHDA lesions did not show any significant reduction compared with the group receiving only 6-OHDA (Fig. 8*D*). The next day, animals were tested for amphetamine-induced circling behavior. Mice receiving 6-OHDA and GDNF-c17.2 grafts showed a 90% reduction in the number of net ipsilateral turns compared with animals receiving 6-OHDA alone, whereas animals receiving the MT-c17.2 grafts and 6-OHDA lesions did not differ from the 6-OHDA group (Fig. 8*E*). Thus, our results are consistent with the prevention of behavioral deficits by GDNF-c17.2 cells in a 6-OHDA model of PD.

#### DISCUSSION

Our study shows that NSCs constitute very useful tools to deliver transgenes with therapeutic value because they locally disperse after grafting, integrate in the host adult brain, and differentiate into multiple, stable phenotypes. Furthermore, we demonstrate that NSCs can stably release high levels of GDNF for at least 4 months, preventing the degeneration of dopaminergic neurons and motor alterations in a mouse model of PD.

#### NSCs as a tool to deliver GDNF in the adult brain

One difficulty often encountered when expressing foreign genes in NSCs has been the downregulation of transgenes during cell differentiation (Flax et al., 1998; Benedetti et al., 2000). However, in the present study, we constructed an expression vector using a previously described  $\beta$ -actin promoter with a cytomegalovirus (CMV) enhancer (Niwa et al., 1991) fused to a bicistronic construct with a selectable marker and a mini-intron. The sustained high levels of transgene expression that we achieve in NSCs with this vector suggests that *ex vivo* gene transfer and grafting of engineered NSCs could constitute a clear alternative to direct



**Figure 8.** GDNF-c17.2 grafted cells prevented the 6-OHDA-induced reduction of TH staining in the striatum and behavioral abnormalities associated with unilateral 6-OHDA lesions. *A–C*, TH immunostaining in sections through the intact striatum (*A*), the 6-OHDA lesioned and MT-c17.2 grafted striatum (*B*), and the 6-OHDA lesioned and GDNF-c17.2 grafted striatum (*C*). *D*, Total net apomorphine-induced rotations contralateral to the lesioned side during 5 min at 10 min after administration. \* $p < 0.01$  versus lesioned substantia nigra as determined by one-way ANOVA (significant effect of treatment,  $p < 0.03$ ;  $F_{(2,15)} = 4.564$ ). *E*, Total net amphetamine-induced rotations ipsilateral to the lesioned side during 3 min at 15, 30, and 45 min after administration. \*\* $p < 0.01$  versus lesioned substantia nigra grafted or not with the MT-c17.2 cell line as determined by one-way ANOVA (significant effect of treatment,  $p < 0.006$ ;  $F_{(2,14)} = 7.549$ ). Scale bar (in *A*): 1 mm.

gene transfer techniques. The delivery of transgenes by NSCs has the advantages that no genetic modification is introduced in the cells of the host, no viral particles have to be introduced in the nervous system, and it allows further engineering of the cells to introduce extra safety features, such as the expression of genes to allow the selective elimination of the NSCs from the host. Moreover, because NSCs can be expanded *in vitro*, they can be extensively characterized and standardized to determine their quality and the efficiency and biological activity of the transgene before grafting.

One other important issue when considering the grafting of cells in the brain as a source for neuroprotective molecules is their survival. Our results show that both GDNF-NSCs and MT-NSCs engraft in the adult brain with very similar efficiency. In both cases, we found more efficient engraftment in nude mice than in wild-type mice, suggesting that, although allografts can survive in the host brain, they are targeted by the immune system. We are currently characterizing the cellular immune response of the host and studying whether standard immunosuppressive therapies can prevent the immune reaction that takes place between 2 weeks and 4 months after allografting. In the future, as an alternative to immunosuppression, strategies based on grafting of multipotent stem cells isolated from the same individual or from donors with compatible antigens could be developed.

With regard to the properties of NSCs after engraftment in the adult striatum, our results indicate that GDNF-c17.2 NSCs are able to integrate and differentiate into stable neural phenotypes. Importantly, the engraftment and differentiation of GDNF-c17.2 NSCs does not affect their ability to maintain stable levels of biologically active GDNF. Moreover, our results suggest that the high levels of GDNF do not affect the ability of the NSCs to

migrate, integrate, and survive within the striatum but seems to affect the fate of the cells derived from them. In our experiments, the only clear effect attributable to GDNF was the increase in the proportion of CNPase-positive oligodendrocytes derived from the GDNF-c17.2 (81%) compared with the control MT-c17.2 (13.5%) cells. Interestingly, these cells predominantly integrated in the white matter tracts within the striatum, together with host oligodendrocytes, suggesting that GDNF expression in c17.2 NSCs might either favor or promote the differentiation of NSCs into oligodendrocytes.

### Neuroprotection by GDNF-NSCs in a model of Parkinson's disease

A multitude of neurotrophic factors have been found to promote the survival or prevent the degeneration of substantia nigra pars compacta dopaminergic neurons. Among them, GDNF is one of the most potent survival factors for these neurons both *in vitro* and in animal models of PD (Lin et al., 1993; Beck et al., 1995; Boewencamp et al., 1995; Kearns and Gash, 1995; Sauer et al., 1995; Tomac et al., 1995; Gash et al., 1996; Åkerud et al., 1999). However, GDNF has also proven to be a potent neurotrophic factor for many other populations of neurons, including motor neurons (Henderson et al., 1994; Oppenheim et al., 1995; Yan et al., 1995) and central noradrenergic neurons (Arenas et al., 1995). Thus, both the potency and the broad spectrum of biological activities of GDNF could make the activation of multiple target cells difficult to prevent. Consistent with this, intracerebroventricular administration of high doses of GDNF has been found to induce adverse effects, including weight loss in rodents (Hoane et al., 1999) and nausea, behavioral disturbances, and weight loss in human patients (Kordower et al., 1999). Thus, alternative local delivery techniques need to be developed to implement a viable therapy with this molecule. In the last years, several approaches, including the local infusion of GDNF protein (Beck et al., 1995; Boewencamp et al., 1995; Kearns and Gash, 1995; Sauer et al., 1995; Tomac et al., 1995; Gash et al., 1996), the implantation of polymer-encapsulated cells releasing GDNF (Lindner et al., 1995), and the viral-mediated GDNF gene transfer (Choi-Lundberg et al., 1997; Mandel et al., 1997, 1999; Bohn et al., 1999; Bensadoun et al., 2000; Kirik et al., 2000; Kordower et al., 2000) have been found to exert neuroprotective and/or neuroregenerative effects on dopaminergic neurons in animal models of Parkinson's disease. Neural stem cells have been successfully engineered to deliver other neurotrophic factors and have been proven to be effective in neuroprotective strategies (Martínez-Serrano and Björklund, 1997); but to our knowledge, the ability of neurotrophic factors released by neural stem cells to prevent the degeneration of adult substantia nigra dopaminergic neurons in a model of Parkinson's disease has not been examined previously. Our results suggest that transplantation of engineered NSCs could be an effective and viable strategy to locally deliver GDNF in the brain, because GDNF-expressing NSCs integrated and differentiated well after grafting, dispersed within, but remained restricted to, the striatum, and maintained GDNF expression for at least 4 months. Moreover, although the dose of cells grafted provided no more than 50 ng/d of GDNF and no accumulation of GDNF was observed in the striatum, we observed retrograde transport of GDNF by substantia nigra dopaminergic neurons and a full biological response to the factor, which resulted in the protection of dopaminergic neurons and the prevention of motor disturbances in the absence of noticeable body weight loss or other adverse effects.

It is important to note that, in the intrastriatal 6-OHDA model that we performed, the degeneration of dopaminergic neurons starts by the terminals and after 2 weeks yields a 60% loss of dopaminergic neurons and a behavioral deficit, as assessed in the apomorphine- and amphetamine-induced rotation tests. This model would correspond in humans to an early-stage PD characterized by a predominant loss of dopaminergic terminals in the striatum, a 50% cell loss in the substantia nigra, and initial motor symptoms (Fearnley and Lees, 1991). In this model, we found increased survival of nigral dopaminergic neurons and improved behavioral performance in animals lesioned with 6-OHDA and grafted with the GDNF-NSCs, which is consistent with a neuroprotective action of GDNF. This result differs from other studies exploring the regenerative properties of GDNF, in that the improvement of the motor performance takes place at later stages, when axons regenerate in response to GDNF (Bensadoun et al., 2000; Kirik et al., 2000). Thus, our results show that administration of GDNF by GDNF-c17.2 cells efficiently prevents the retrograde degeneration of dopaminergic axons, the loss of dopaminergic neurons, and the early motor deficits associated to them.

In conclusion, the experiments presented here demonstrate that NSCs can be efficiently engineered to deliver therapeutic levels of transgenes to target tissues for at least 4 months *in vivo*. Moreover, NSCs engineered to deliver GDNF were found to prevent the degeneration of dopaminergic neurons and the behavioral impairment in a model of PD. In such a way, our results demonstrate that GDNF-NSCs are particularly effective at protecting dopamine neurons in a model of PD and suggest that strategies based on the local delivery of neurotrophic factors by NSCs may find an application in the treatment of Parkinson's disease.

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## Identification of the $\alpha_6$ Integrin as a Candidate Receptor for Papillomaviruses

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Papillomaviruses (PVs) bind in a specific and saturable fashion to a range of epithelial and other cell lines. Treatment of cells with trypsin markedly reduces their ability to bind virus particles, suggesting that binding is mediated via a cell membrane protein. We have investigated the interaction of human PV type 6b L1 virus-like particles (VLPs) with two epithelial cell lines, CV-1 and HaCaT, which bind VLPs, and a B-cell line (DG75) previously shown not to bind VLPs. Immunoprecipitation of a mixture of PV VLPs with [<sup>35</sup>S]methionine-labeled cell extracts and with biotin-labeled cell surface proteins identified four proteins from CV-1 and HaCaT cells of 220, 120, 87, and 35 kDa that reacted with VLPs and were not present in DG75 cells. The  $\alpha_6\beta_4$  integrin complex has subunits corresponding to the VLP precipitated proteins, and the tissue distribution of this complex suggested that it was a candidate human PV receptor. Monoclonal antibodies (MAbs) to the  $\alpha_6$  or  $\beta_4$  integrin subunits precipitated VLPs from a mixture of CV-1 cell proteins and VLPs, whereas MAbs to other integrin subunits did not. An  $\alpha_6$  integrin-specific MAb (GoH3) inhibited VLP binding to CV-1 and HaCaT cells, whereas an anti- $\beta_4$  integrin MAb and a range of integrin-specific and other MAbs did not. Furthermore, human laminin, the natural ligand for the  $\alpha_6\beta_4$  integrin, was able to block VLP binding. By use of sections of monkey esophagus, the distribution of  $\alpha_6$  integrin expression in the basal epithelium was shown to coincide with the distribution of bound VLPs. Taken together, these data suggest that VLPs bind specifically to the  $\alpha_6$  integrin subunit and that integrin complexes containing  $\alpha_6$  integrin complexed with either  $\beta_1$  or  $\beta_4$  integrins may act as a receptor for PV binding and entry into epithelial cells.

Human papillomaviruses (HPVs) are recognized as the etiological agents for skin, plantar, genital, and laryngopharyngeal warts, and infection with some genital genotypes of HPV is a major risk factor for the development of anogenital cancer (3, 40). The inability to propagate PV in vitro has hampered definition of the early phases of PV infection. PV infects skin and mucosal surfaces, and this restricted cell tropism is thought to be mediated by PV interaction with host cell factors. The first step in infection with a crystalline virus is generally attachment of the virus to a specific receptor on the surface of a susceptible host cell. The receptor molecule on the cell surface can be a protein (usually a glycoprotein) or a carbohydrate structure localized on glycoproteins or glycolipids. For most viruses, attachment to cell membranes via an interaction with cell surface proteins is the primary determinant of tropism (12), and many viral receptors are now well characterized. Echoviruses 1 and 8 use the integrin  $\alpha_2\beta_1$  (VLA-2) (1); the  $\alpha_v\beta_3/\alpha_5$  integrins function as a secondary receptor for adenovirus (36); cytomegalovirus (24) and simian virus 40 (4) bind to major histocompatibility complex (MHC) class I proteins; and human immunodeficiency virus binds to CD4 via gp120 (7). However, the nature of any putative cellular receptor for PV has yet to be elucidated.

The PV capsid consists of two virally encoded proteins, L1 and L2, which migrate in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 55 and 75 kDa, respectively (9, 19). The L1 protein is the major capsid protein and when expressed in various eukaryotic expression systems is

able to self-assemble into virus-like particles (VLPs) (15, 28, 34, 39). The L2 protein appears to be involved in DNA binding and encapsidation (38). VLPs have been used to characterize PV binding to cells by various techniques, and we and others have shown that PV particles bind to an unknown receptor present in a variety of different cell types (21, 25, 26, 35). VLP binding is trypsin sensitive, suggesting that the putative receptor is a protein, and binding has been shown to be saturable with  $1 \times 10^4$  to  $2 \times 10^4$  receptors/cell (25, 35). Soluble L1 protein not configured as VLPs also binds to cells but in a trypsin-insensitive and nonsaturable manner (25). VLPs composed of either L1 alone or L1 and L2 have been shown to be equally effective in binding to cells (26). VLPs bind to a wide range of cell types, but the highest degree of binding per cell occurs with epithelial and mesenchymal cells. Together, these data suggest that any putative receptor is a conserved and widely expressed molecule.

It has previously been shown that a human B lymphoma cell line (DG75) did not bind VLPs (25). We now demonstrate that four VLP-binding proteins of 220, 120, 87, and 35 kDa are found in two cell lines that bind VLPs but not in DG75 cells. As two of these proteins possessed the same mass as the  $\alpha_6\beta_4$  integrin complex (220 and 120 kDa), this complex was investigated as a potential receptor candidate.

### MATERIALS AND METHODS

VLPs, cell lines, and antibodies. HPV type 6b (HPV6b) L1 VLPs were produced in SF9 cells by using HPV6b L1 recombinant baculovirus, purified, quantitated, and examined by electron microscopy as previously described (25). CV-1 African green monkey kidney epithelial cells were obtained from the American Type Culture Collection (catalog no. CCL70); HaCaT human epithelial cells were a gift from Norbert Fusenig, Deutsches Krebsforschungszentrum, Heidelberg, Germany; and DG75, a Burkitt's lymphoma line, was a gift of Ihor Misko, Queensland Institute of Medical Research, Brisbane, Queensland, Australia.

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Monoclonal antibodies (MAbs) 8 and 39, raised to HPV16 L1, which cross-react with HPV6b L1, were a gift of Stephen Kelsall, Department of Microbiology, University of Western Australia. The following MAbs to integrins were purchased: anti-integrin  $\alpha_6$  intracytoplasmic domain (4E9G8; Immunotech, Marseille, France), anti-integrin  $\alpha_6$  extracellular domain (GoH3; Serotec Ltd., Oxford, England), and anti- $\beta_4$  integrin (3E1; Gibco-BRL). Anti- $\alpha_5$  (P3G8), anti- $\alpha_5\beta_5$  (P5H9-E11), anti- $\alpha_2$  (P1E6), anti- $\alpha_4$  (P4G9-E11), anti- $\beta_1$  (P5D2), and anti- $\beta_2$  (P4H9-A11) integrin antibodies were a kind gift from Elizabeth Wagner, University of Minnesota. An HPV6b L1 VLP-specific rabbit polyclonal antiserum, raised by immunizing a rabbit with purified HPV6b L1 VLPs three times without adjuvant, was a gift of Shi Wen Peng, Centre for Immunology and Cancer Research (CICR), Brisbane, Queensland, Australia. This antiserum recognizes native but not denatured VLPs by enzyme-linked immunosorbent capture assay. Horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit immunoglobulin (Ig) antisera were purchased from Silenus (Hawthorne, Australia), and fluorescein isothiocyanate (FITC)-conjugated anti-mouse and anti-rabbit Ig antisera were purchased from Sigma (Raritan, N.J.) and Rockland (Gilbertsville, Pa.). The CD34-specific MAb BI-3C5 was a gift from Robert Tindie, CICR, Brisbane, Queensland, Australia, and the MHC class I MAb was from Serotec. Mouse laminin (Boehringer Mannheim), human vitronectin (Sigma), and RGD motif peptides (GRGDSP and GRGESF) (Life Technologies, Gaithersburg, Md.) were used as purchased. Human laminin was purchased from Life Technologies.

**[<sup>35</sup>S]methionine labeling of cells.** Cells ( $2 \times 10^7$ ) were washed twice in methionine-free RPMI (CSL Ltd., Melbourne, Victoria, Australia) and incubated in the same medium for 30 min at 37°C. The cells were labeled with [<sup>35</sup>S]methionine (100  $\mu$ Ci/ml; Amersham) for 3 h, washed twice with ice-cold 0.15 M phosphate-buffered 0.9% saline (PBS) (pH 7.4), scraped off, and resuspended in homogenization buffer (100 mM HEPES [pH 7.9], 20 mM MgCl<sub>2</sub>, 200  $\mu$ M CaCl<sub>2</sub>, 100 mM NaF, 200  $\mu$ M phenylmethylsulfonyl fluoride, and 100  $\mu$ g aprotinin per ml). The cells were lysed on ice with 300 strokes of a Dounce homogenizer.

**Biotinylation of VLPs and cell surface proteins.** VLPs were biotinylated at a molar ratio of 1,000:1 with Immuno Sulfo-NHS-biotin (Pierce, Rockford, Ill.). VLPs (20  $\mu$ g) were mixed with biotin so that the final biotin concentration was 500 ng/ml and held at room temperature for 4 h. Biotin was then removed either by centrifugation through Centricon microconcentrators (Amicon) or by dialysis against PBS overnight. Cell surface proteins were biotinylated by washing  $5 \times 10^7$  cells three times in ice-cold PBS containing 0.1 mM CaCl<sub>2</sub>-1 mM MgCl<sub>2</sub> (PBS-CaMg). N-Hydroxysulfosuccinimide (NHS)-biotin (sulfosuccinimidobiotin) was added to a final concentration of 200  $\mu$ g/ml in PBS-CaMg, and the cells were held for 30 min at 4°C. The biotin solution was removed, fresh biotin solution was added, and the cells were held for a further 30 min at 4°C. The cells were washed once with PBS containing 0.1 M glycine and four times with PBS before being harvested by centrifugation or scraping.

**Immunoprecipitations.** Cell extracts were prepared by lysis of cells in RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.14 mg of aprotinin per ml, Tris-HCl; pH 7.5) followed by brief sonication. Extracts were clarified by centrifugation and pretreated with anti-VLP antibody and protein A-Sepharose (Pharmacia, Uppsala, Sweden) for 60 min at 4°C to remove cross-reacting proteins. Cell extract (70  $\mu$ g) was made up to 500  $\mu$ l in PBS before the addition of 5  $\mu$ g of VLPs, and the mixture was held at 4°C for 60 min. Anti-VLP antibodies were added, and the mixture was held at 4°C for 30 min. Protein A-Sepharose, which had previously been blocked in 5% skim milk-1% bovine serum albumin in PBS, was added, and the mixture was held at 4°C for a further 30 min. Complexes were washed five times at 4°C with 0.5 ml of PBS before the addition of SDS-PAGE loading buffer. Samples were boiled for 5 min, briefly centrifuged, and subjected to SDS-PAGE.

**Flow cytometry.** Analysis of expression of cell surface molecules by flow cytometry was performed as described previously (25).

**VLP binding assays.** HaCaT or CV-1 cells ( $10^5$ ) were grown to confluence as adherent monolayers, detached by using 0.25% trypsin-0.02% EDTA, and then held in suspension for 2 h at 37°C with occasional swirling as described previously (25). Before analysis of binding, cells were first washed twice with Dulbecco modified Eagle medium plus 0.2% bovine serum albumin (binding medium) and then held in binding medium for 1 h on ice, in a total volume of 200  $\mu$ l, with added antibodies or receptor ligands as indicated. The cells were then washed twice with binding medium by being spun for 20 s at  $9,500 \times g$ . VLPs (50 to 150 ng, suspended in binding medium) were added to the cells, which were held on ice for 90 min. The cells were extensively washed with PBS, resuspended in SDS-PAGE loading buffer, boiled for 10 min, and loaded onto an SDS-polyacrylamide gel. For studies of neutralization of HPV6b VLPs, VLPs were mixed with a 1:12 dilution of a rabbit polyclonal anti-HPV6b VLP antibody in binding medium for 1 h at 22°C and then added to cells.

**Immunoblotting.** Proteins separated by SDS-PAGE (10% resolving gel, 4.5% stacking gel, 60 min at 100 V) were electrically transferred (80 min at 200 V) to nitrocellulose filters (Amersham) and held in PBS-0.05% Tween 20 plus 5% milk powder for 1 h at 22°C. Antibodies were added, and the filter was held for 1 h at 22°C. After three washes with PBS plus 0.05% Tween 20, bound antibody was visualized by incubation with HRP-conjugated anti-species antiserum for 1 h at 22°C, followed by three washes as described above and HRP detection by

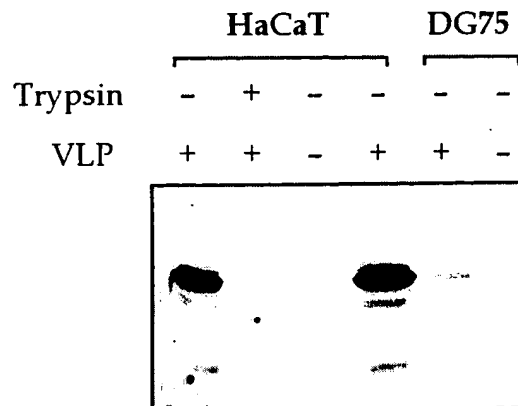


FIG. 1. Binding of HPV6b L1 VLPs to cells detected by immunoblotting for L1 protein using L1-specific MAb. HaCaT and DG75 cells were treated with trypsin and exposed to VLPs as indicated (+, treatment; -, no treatment) and washed extensively. The cells were then lysed, and the lysate was separated by SDS-PAGE and transferred to nitrocellulose, which was exposed to L1 MAb 39. Bound antibody was detected by use of HRP-conjugated antiserum to mouse IgG followed by ECL. The major immunoreactive L1 band has an apparent molecular mass of 55 kDa.

enhanced chemiluminescence (ECL) (Amersham) according to the manufacturer's instructions. The intensity of immunoblot bands was assessed with a Molecular Dynamics Personal Densitometer.

**Immunofluorescence.** Monkey esophageal skin sections (Inova, San Diego, Calif.) were exposed to VLPs suspended at 50 ng/ml in PBS for 60 min at 22°C and washed twice in PBS. The slides were then exposed to a conformational anti-VLP rabbit antiserum (or an anti-integrin antibody), washed, and held for a further 60 min at room temperature with an appropriate FITC-conjugated anti-Ig antibody before detection by epifluorescence microscopy, using appropriate filter combinations for FITC.

## RESULTS

**Characterization of HPV VLP receptor-negative and -positive cell lines.** It has previously been shown that HPV6b L1 VLPs bind to a range of cell lines in a specific, saturable manner. A monkey epithelial cell line, CV-1, was shown to bind the most VLPs per cell of the cell lines tested, while a human B-cell line, DG75, was unable to bind VLPs (25). To increase the sensitivity of our VLP binding assay, we modified the previously used assay, in which VLP binding was detected by flow cytometry, to allow detection of VLP binding by immunoblotting with an L1-specific MAb. SDS-PAGE-separated extracts of cells exposed to VLPs were probed with the MAb, and VLP binding was quantitated by densitometry. When VLP binding was assayed by using a monolayer cell culture, some assay conditions promoted cell detachment, so assays were routinely performed using cells in suspension culture. Where assay conditions permitted comparison, the same results could be obtained for cells grown in suspension and in monolayers (data not shown).

Using this modified VLP binding assay, we confirmed the observation from the flow cytometry-based assay that HPV6b L1 VLPs bound to CV-1 cells, whereas binding to DG75 cells was minimal (Fig. 1). We observed that the human epithelial cell line HaCaT bound similar amounts per cell of HPV6b VLPs to CV-1 cells. Binding of VLPs to CV-1 and many other cell types is prevented by pretreatment of the cells with trypsin (21, 25), suggesting that a membrane protein is involved in VLP binding to cells. We therefore examined whether binding of VLPs to HaCaT cells was trypsin sensitive (Fig. 1) and observed, using the modified VLP binding assay, that trypsin treatment of HaCaT cells prior to exposure to VLPs reduced

binding of VLPs by 90%, in agreement with the estimate of 88% reduction from flow cytometry analysis. The binding of VLPs to trypsin-treated HaCaT cells and the minimal binding of VLPs to DG75 cells observed in the more sensitive assay used here are likely to represent binding of denatured L1 molecules, which adhere nonspecifically to cells (25): approximately 10% of our VLP preparation has an elution profile on size exclusion gel chromatography consistent with monomeric, presumably denatured, L1 protein. Having shown that CV-1 and HaCaT cells bind VLPs similarly, and having confirmed in a more sensitive assay that DG75 cells did not bind a significant amount of VLPs at a concentration (50 ng/ml) sufficient to saturate the saturable binding of VLPs to CV-1 cells, we used these three cell lines to identify the trypsin-sensitive membrane proteins which mediate saturable binding of HPV6b L1 VLPs to cells. Hereafter, such proteins are referred to as PV receptor proteins.

**Characterization of epithelial cell proteins that interact with VLPs.** Our first approach to identification of PV receptor proteins was to enrich [ $^{35}$ S]methionine-labeled total cell protein extracts for proteins which could bind to VLPs, using coprecipitation. Cell extracts were exposed to HPV6b L1 VLPs, and receptor protein-VLP complexes were isolated by immunoprecipitation with HPV16 L1-specific MAb 39, which binds HPV6b L1 VLPs. Proteins with apparent molecular masses of 120 and 220 kDa as determined by SDS-PAGE were consistently enriched in immunoprecipitates from CV-1 cell lysates mixed with HPV6b L1 VLPs (Fig. 2A), whereas no proteins of similar size could be isolated from DG75 cell lysates by the same technique.

To confirm and extend these initial findings and to increase the probability of defining membrane-associated PV receptor proteins, we labeled cell surfaces of CV-1 and DG75 cells with NHS-biotin, solubilized the cell membrane with radioimmuno-precipitation assay buffer and mixed the solubilized membrane proteins with HPV6b VLPs. Proteins binding to VLPs were identified by immunoprecipitation of the VLP-membrane protein mixture with L1-specific MAbs as before, followed by analysis of the biotinylated proteins in the precipitate by SDS-PAGE. While several cell surface proteins from both DG75 and CV-1 cells (Fig. 2B) were reactive, four biotinylated membrane proteins were consistently identified in CV-1 cell extracts immunoprecipitated with an L1 MAb in the presence of VLPs that were not identified when either DG75 extracts or CV-1 extracts immunoprecipitated without addition of VLPs were used. These proteins had apparent molecular masses of 220, 120, 87, and 35 kDa as determined by SDS-PAGE. Two proteins (220 and 120 kDa) were of comparable size to the proteins identified as candidate PV receptor proteins in [ $^{35}$ S]methionine-labeled protein extracts.

**Distribution of the  $\alpha_6\beta_4$  integrin.** The SWISS-PROT protein sequence database was searched for epithelial cell surface proteins with apparent molecular masses corresponding to those of the proteins apparently binding to VLPs. This search indicated that the  $\alpha_6\beta_4$  integrin was a candidate receptor protein, as the  $\alpha_6$  integrin subunit is a heterodimer of a 120- and a 25-kDa protein, while the  $\beta_4$  subunit is a single protein of 220 kDa. Tissue distribution of the  $\alpha_6\beta_4$  integrin is limited to epithelial, endothelial, and neural cells (14, 30), which is consistent with the distribution of cell types that show the highest affinity for VLPs (25). We therefore investigated expression of the  $\alpha_6\beta_4$  integrin on CV-1 and DG-75 cells by flow cytometry. CV-1 cells expressed both  $\alpha_6$  and  $\beta_4$  integrins as membrane proteins, while DG75 cells expressed neither (Fig. 3). As expected, both cell types expressed the  $\beta_1$  integrin chain (a 130-

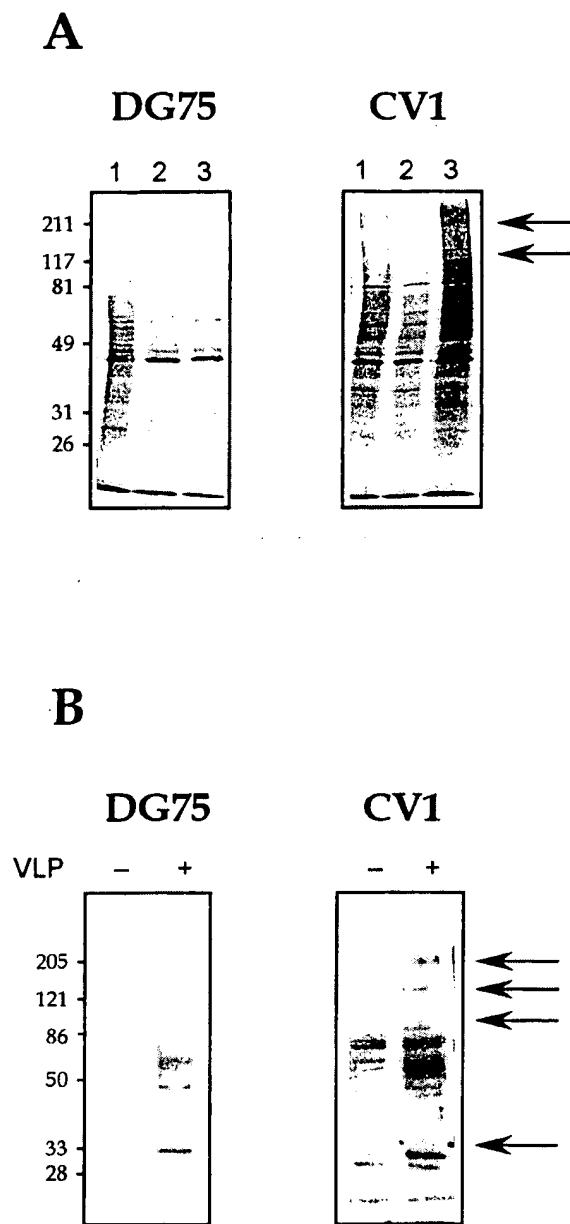


FIG. 2. Cellular proteins that bind to HPV6b L1 VLPs. (A) [ $^{35}$ S]methionine-labeled CV-1 and DG75 cell extracts were exposed to HPV6b L1 VLPs, and VLP-binding proteins were immunoprecipitated as indicated. Precipitates were separated by SDS-PAGE and analyzed by autoradiography. Lanes 1, no anti-serum and no VLPs; lanes 2, anti-L1 MAb 39; lanes 3, as for lane 2 but with VLPs added. Bands specific for CV-1 cells precipitated with L1 VLP-specific antiserum are indicated (arrows). (B) DG75 and CV-1 cell surface proteins were biotin labeled. Cell extracts were mixed with VLPs, and interacting proteins were precipitated with a VLP-specific antiserum, separated by SDS-PAGE as for panel A, and transferred to nitrocellulose. Biotinylated proteins were detected by use of peroxidase-labeled streptavidin followed by ECL. Four bands (arrows) of 220, 120, 87, and 35 kDa were observed only in CV-1 lysates precipitated in the presence of VLPs. + and -, addition or no addition of VLPs, respectively. Molecular masses (in kilodaltons) are shown on the left.

kDa protein that is able to bind to many  $\alpha$ -integrin chains, including  $\alpha_6$ ).

**Association of VLPs with integrin subunits.** To determine whether  $\alpha_6\beta_4$  integrin could associate with HPV VLPs, we

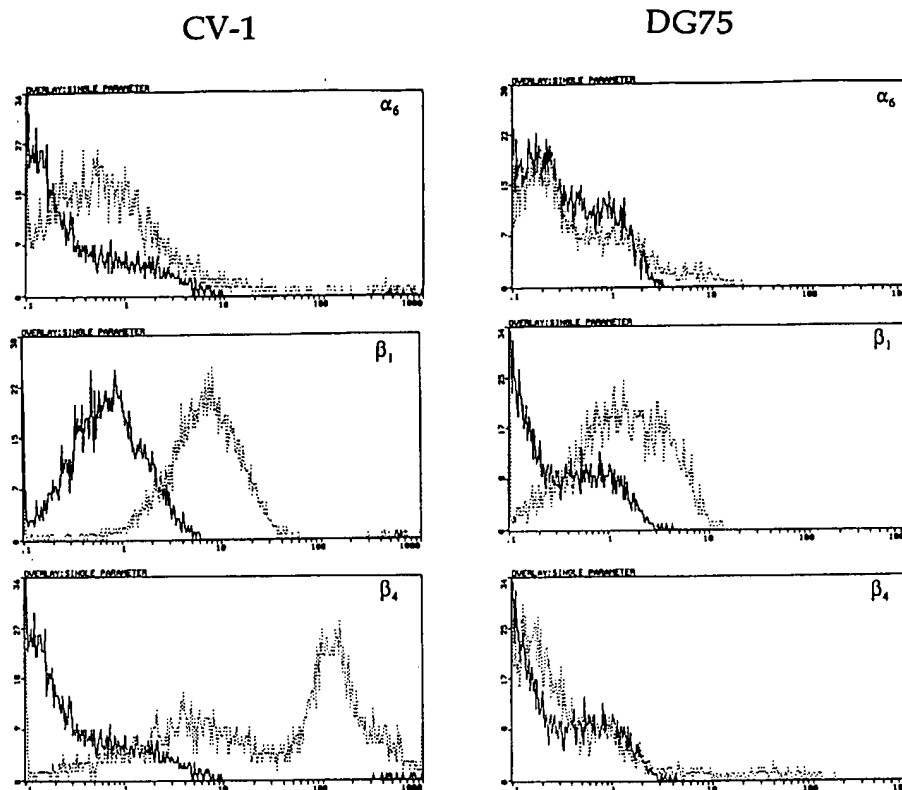


FIG. 3. Analysis of the expression of  $\alpha_6$ ,  $\beta_1$ , and  $\beta_4$  integrins on CV-1 and DG75 cells by flow cytometry. In each panel, the black line corresponds to the histogram of fluorescence intensities for cells exposed to secondary antibody only and the grey line indicates cells exposed additionally to the indicated primary antibody (top panels,  $\alpha_6$ ; middle panels,  $\beta_1$ ; bottom panels,  $\beta_4$ ).

performed coimmunoprecipitation experiments, using biotinylated VLPs to facilitate detection of precipitated VLPs. We first established that VLP biotinylation did not affect VLP morphology or VLP binding to cells. As shown in Fig. 4, biotinylated VLPs had a normal appearance under electron microscopy and were able to bind cells with an affinity equal to that of nonbiotinylated VLPs. As further confirmation that VLP biotinylation did not interfere with receptor interaction, we established that binding of biotinylated VLPs to cells was blocked by a polyvalent antibody recognizing conformational epitopes of HPV6b VLPs and previously shown to block VLP binding to epithelial cells (data not shown). Furthermore, biotinylated VLPs were able to react with proteins of 220, 120, 87, and 35 kDa from CV-1 cell extracts labeled with [ $^{35}$ S]methionine (data not shown). Biotinylated VLPs were used in coimmunoprecipitation experiments with CV-1 and HaCaT membrane proteins as before. Precipitation of VLP-membrane protein complexes was examined, using a range of MABs specific for integrin proteins. Both  $\alpha_6$ -specific (Fig. 5, lane 6) and  $\beta_4$ -specific (Fig. 5, lane 9) MABs precipitated a protein from mixtures of VLPs and CV-1 membrane protein with the same apparent molecular mass as a protein precipitated by an anti-L1 MAB known to react with HPV6b VLPs (Fig. 5, lane 4). This protein was not identified in unprecipitated VLP-membrane protein mixtures (Fig. 5, lane 3) or in immunoprecipitates obtained with MABs specific for a range of other integrins (anti- $\alpha_2$ , - $\beta_1$ , - $\alpha_4$ , and - $\beta_2$ ) or for CD34, a cell membrane protein present on DG75 but not CV-1 cells. All MABs used were precipitated effectively by protein A-Sepharose, as judged

by the quantity of immunoreactive mouse IgG within the immunoprecipitate (data not shown).

**Antibodies to  $\alpha_6$  integrin reduce VLP binding to cells.** To confirm a role for the  $\alpha_6$  integrin chain in the binding of HPV VLPs to cells, we determined whether VLP binding to cells could be inhibited by pretreatment of the cells with integrin chain-specific MABs. For these assays, HaCaT cells, which express the  $\alpha_6\beta_4$  integrin (16), were used. Pretreatment of HaCaT cells with an  $\alpha_6$ -specific MAB, GoH3, reduced VLP binding by an average of  $(63 \pm 6.3)\%$  (Fig. 6B). Figure 6B shows the averages from 10 separate experiments. An anti- $\beta_4$  antibody reduced binding only slightly (18%). Other integrin MABs, specific for subunits  $\beta_1$ ,  $\alpha_2$ ,  $\alpha_v$ , and  $\alpha_v\beta_5$ , did not significantly reduce VLP binding (15, 9, 13, and 5%, respectively), nor did antibodies against MHC class I framework determinants (4%) or CD34 (13%) (Fig. 6B). The same results were observed with CV-1 cells as with HaCaT cells. No increase in blocking of VLP binding over that observed with an  $\alpha_6$ -specific MAB could be demonstrated if a combination of  $\alpha_6$ - and  $\beta_4$ -specific MABs or of  $\alpha_6$ - and  $\beta_1$ -specific MABs was used. The natural ligand for the  $\alpha_6\beta_4$  heterodimer is believed to be laminin. Preincubation of cells with human laminin was able to block binding of VLPs in a dose-dependent manner, with 250 ng having no effect upon binding while 500 ng resulted in complete loss of binding (Fig. 6C). We looked for a reduction of VLP binding to cells by pretreatment of cells with vitronectin or with a polyclonal antiserum specific for the vitronectin receptor ( $\alpha_v\beta_3/\alpha_v\beta_5$ ), but no inhibition of binding was found in either case. Additionally, no reduction in VLP binding was

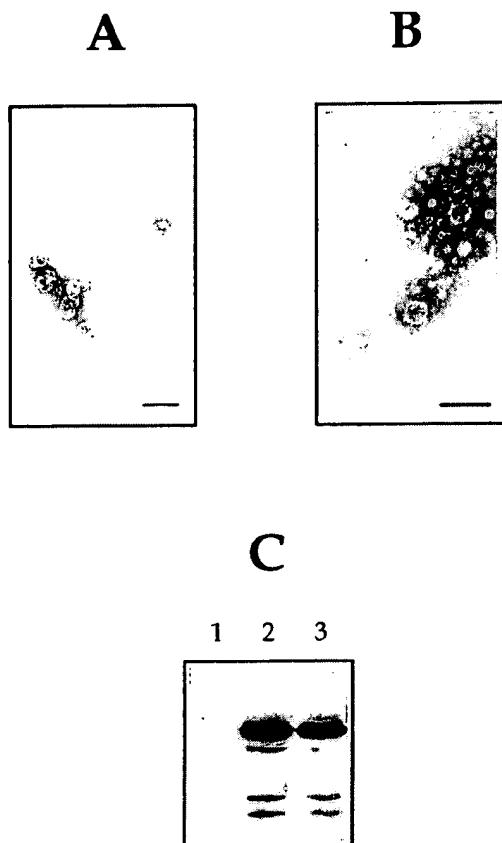


FIG. 4. Biotinylated VLPs. Electron micrographs of normal (A) and biotinylated (B) VLPs (magnifications,  $\times 60,000$  and  $\times 80,000$ , respectively), showing preservation of 55-nm empty PV-like structures; occasional tube forms are seen. Bar, 100 nm. (C) Binding of VLPs to CV-1 cells, using an immunoblot assay for L1 to detect bound VLPs (see Materials and Methods). Lane 1, no VLPs added; lane 2, untreated VLPs; lane 3, biotin-labeled VLPs. In each case, the major band at 55 kDa corresponds to L1 protein.

seen when cells were pretreated with an RGD peptide, which has been demonstrated to inhibit binding of substrates to those integrins which utilize the RGD binding motif, including  $\alpha_v\beta_3/\alpha_v\beta_5$ .

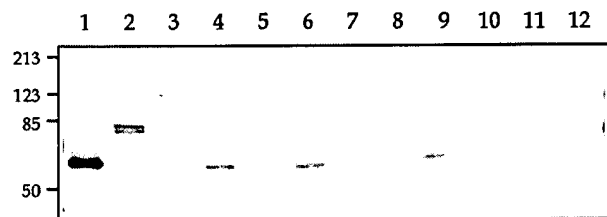


FIG. 5. Interaction between VLPs and integrins. Biotin-labeled VLPs were mixed with digitonin-prepared cell lysates and incubated for 1 h. Immune complexes were precipitated with antibodies (lanes 4 to 12) using protein A-Sepharose. Immune complexes were analyzed by SDS-PAGE and transferred to nitrocellulose, and biotinylated L1 protein, corresponding to bound VLPs, was detected with peroxidase-labeled streptavidin. Lane 1, biotin-labeled VLPs (positive control); lane 2, total cell extract (negative control); lane 3, no antibody; lane 4, anti-L1 MAb; lane 5, no antibody; lane 6, anti- $\alpha_6$  (4E9G5); lane 7, anti- $\alpha_2$ ; lane 8, anti- $\beta_1$ ; lane 9, anti- $\beta_4$ ; lane 10, anti- $\alpha_4$ ; lane 11, anti- $\beta_2$ ; lane 12, anti-CD34. The 80-kDa bands in lane 2 represent streptavidin binding activity in unfractionated cell lysate. Molecular masses (in kilodaltons) are shown on the left.

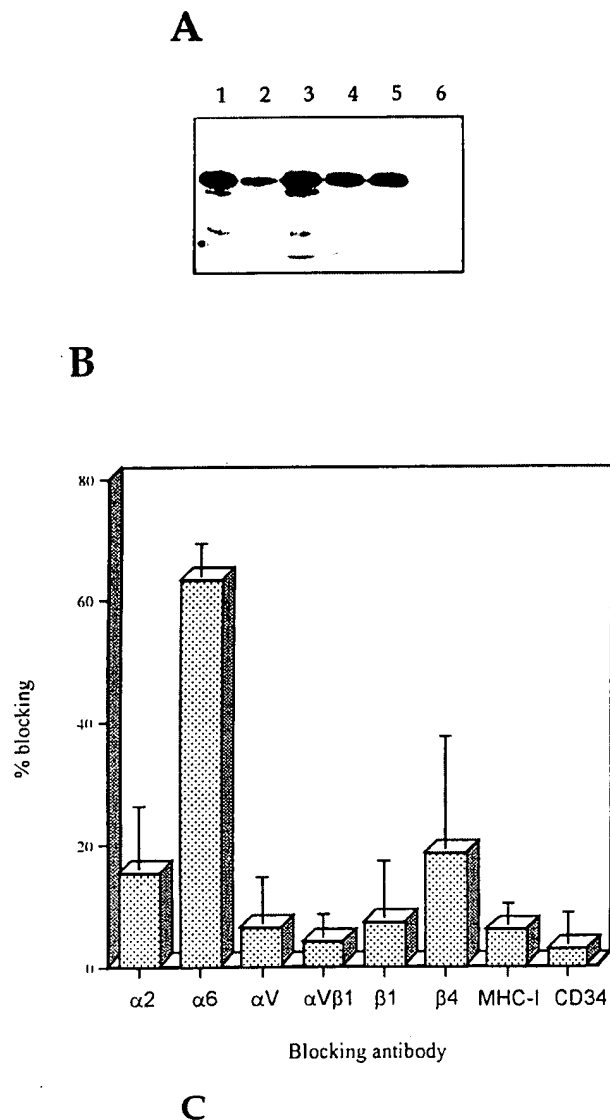


FIG. 6. Blocking of VLP binding to HaCaT cells by integrin antibodies and laminin. (A) Assay as described for Fig. 1, with antibody added to the cells prior to addition of VLPs as indicated below. Lane 1, no antibody; lane 2, anti- $\alpha_6$  (GoH3); lane 3, anti- $\beta_1$ ; lane 4, anti- $\beta_4$ ; lane 5, anti- $\alpha_2$ ; lane 6, cells only (no VLPs added). (B) Reduction of VLP binding by anti-integrin antibodies. Assays were done as for panel A, with binding quantitated by densitometry. Binding to HaCaT cells not pretreated with antibody was arbitrarily assigned a value of 100%. Results for binding to cells treated with each antibody are calculated as a percentage of the binding observed with untreated HaCaT cells and displayed as percent blocking, defined as  $100\% - \text{observed binding}$ . (C) Blockage of VLP binding by human laminin. VLP binding assays were performed as described above, but cells were preincubated as described. Lane 1, cell-only control; lane 2, no antibody; lane 3, anti- $\alpha_6$  (GoH3); lanes 4 and 5, human laminin (250 and 500 ng, respectively).

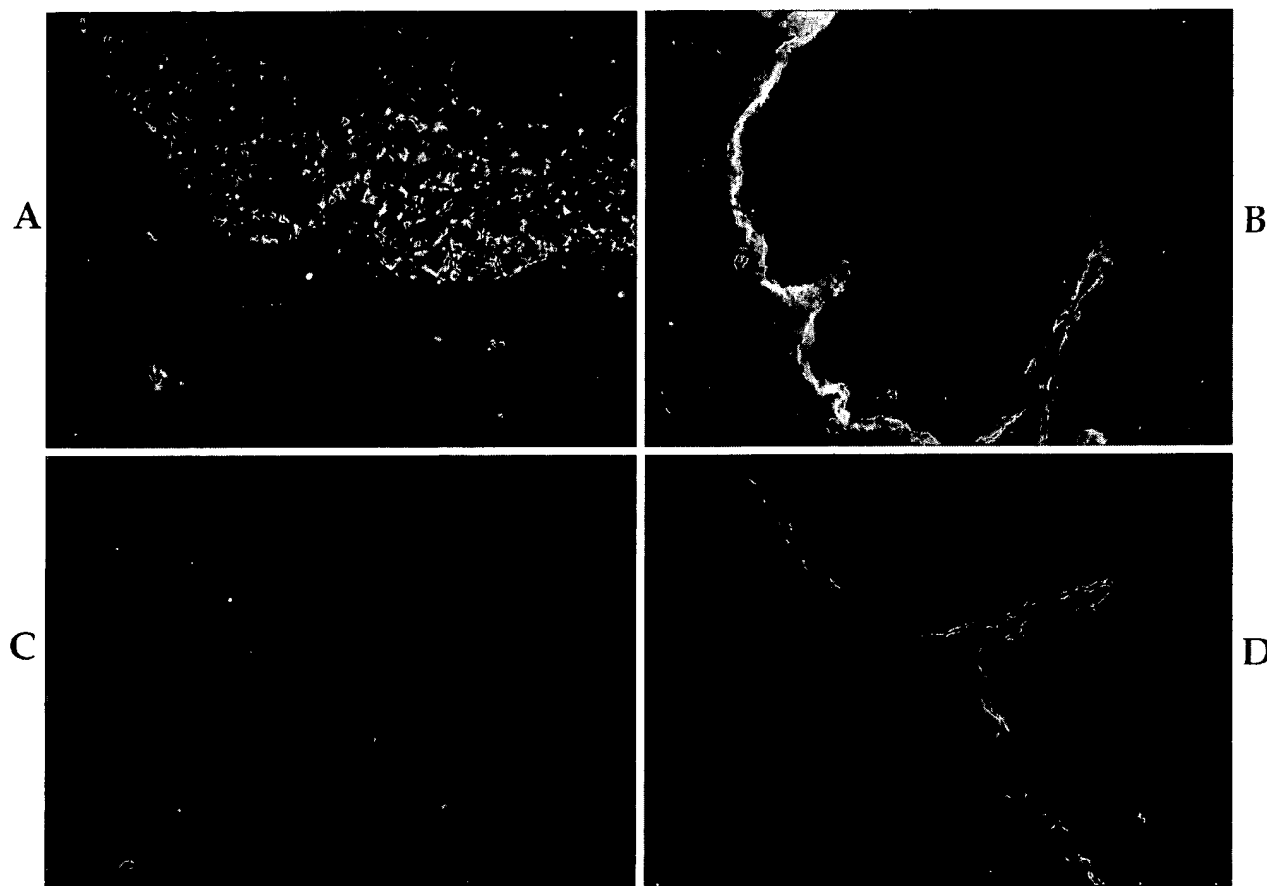


FIG. 7. Distribution of integrin expression and VLP binding in monkey esophageal mucosa. Frozen sections of esophagus were incubated with HPV6b L1 VLPs and rabbit anti-VLP antiserum (A), anti- $\alpha_6$  MAb (B), anti- $\beta_1$  MAb (C), and anti- $\beta_4$  MAb (D). Binding of MAbs for panels B to D was detected with an FITC-labeled anti-mouse IgG antibody. Control sections without primary antibody or without VLPs showed no staining. In each panel, the esophageal lumen is in the upper right. Epifluorescence microscopy with a magnification of ca.  $\times 300$  was used.

**In situ VLP binding to epithelial cells.** Our data obtained with cultured cell lines suggested that the  $\alpha_6$  integrin subunit might be a receptor for HPV VLPs. To investigate whether HPV binding and  $\alpha_6$  integrin expression occur on the same cells in vivo, we examined binding of VLPs and distribution of  $\alpha_6$  integrin in monkey esophageal sections, as we had already determined that a monkey epithelial cell line (CV-1) bound VLPs. On monkey esophageal sections, VLPs bound to basal and immediately suprabasal epithelial cells, and, in concurrence with the hypothesis that  $\alpha_6$  is a component of a VLP receptor, the distribution of staining for  $\alpha_6$  integrin was similar to the distribution of bound VLPs (Fig. 7A versus B). Intense staining for  $\alpha_6$  integrin was observed along the basal aspect of cells along the basement membrane, with lighter staining of the lateral side of basal cells and the first four or five layers of suprabasal cells (Fig. 7B). As expected,  $\beta_4$  integrin staining was limited to the basement membrane cells (Fig. 7D), while  $\beta_1$  integrin expression was distributed throughout the papillary dermis, basement membrane, and first one or two layers of the basal cells (Fig. 7C). All controls were negative for staining (not shown).

## DISCUSSION

Investigation of putative HPV receptors has been hampered by a lack of in vitro replication systems for this virus. Produc-

tion of VLPs in eukaryotic expression systems using recombinant DNA technology has allowed examination of the initial steps in HPV infection. We show here, using a number of different assays, that  $\alpha_6$  integrin is able to interact with VLPs, suggesting that it may function as a cellular receptor for PVs. A protein with a mass similar to that of the  $\alpha_6$  integrin subunit copurifies from cell extracts with VLPs, antibodies to  $\alpha_6$  integrin can precipitate VLPs from a mixture of VLPs and cell extract, a MAb against the  $\alpha_6$  integrin subunit reduces binding of VLPs to cells by 63%, and the distribution of expression of  $\alpha_6$  integrin in tissue sections matches the distribution of bound VLPs. Furthermore, VLPs do not bind to the  $\alpha_6$  integrin-negative B-cell line DG75.

In our studies,  $\alpha_6$ -specific antibodies were able to block VLP binding, whereas antibodies to a range of  $\beta$  integrins were unable to block binding, suggesting that the  $\beta$  chains do not play a key role in VLP interactions with integrins. The integrins are heterodimeric glycoproteins comprising  $\alpha$  and  $\beta$  subunits, which are expressed in a variety of cell types. They are involved primarily in cell-matrix and cell-cell interactions (13). The  $\alpha_6$  subunit has a more limited tissue distribution than most integrins and associates preferentially with the  $\beta_4$  subunit in epithelial cells (14) and the  $\beta_1$  subunit in mesenchymal tissues (30). The  $\beta_4$  subunit is known to form a heterodimer only with the  $\alpha_6$  subunit (13). Furthermore,  $\alpha_6$  combines more efficiently

with  $\beta_4$  than with  $\beta_1$  (10), and in HaCaT cells as well as primary human keratinocytes the  $\alpha_6$  subunit is associated primarily with  $\beta_4$  (16, 29). Our coprecipitation data show that VLPs complex with both  $\alpha_6$  and  $\beta_4$ , whereas anti- $\beta_1$  antibodies were not able to precipitate VLPs from CV-1 cell extracts. However, in HaCaT cells, the higher affinity of  $\beta_4$  for  $\alpha_6$  leaves no  $\alpha_6$  available to pair with  $\beta_1$  (16), and this may be true also for CV-1 cells, as the  $\alpha_6$  integrin subunit associates preferentially with the  $\beta_4$  subunit in epithelial and neuroepithelial cells (14, 30). Mesenchyma-derived cells express  $\alpha_6\beta_1$  but not  $\alpha_6\beta_4$ . It has previously been observed that VLPs bind cells of mesenchymal origin (25) and also that at least two cell lines that express  $\alpha_6\beta_1$  but not  $\alpha_6\beta_4$  were able to bind VLPs. Others have shown that CHO cells, another cell line of mesenchymal origin, can bind VLPs (26, 35). We therefore conclude that VLPs will bind to  $\alpha_6$  whether paired with  $\beta_1$  or  $\beta_4$ . It remains to be determined which complex has higher affinity for VLPs.

The  $\alpha_6$  antibody (GoH3) used in our VLP binding assay did not completely block VLP binding, even at a large molar excess with respect to  $\alpha_6$  complexes and VLPs. However, an average reduction in binding of 63% compares favorably with figures for other viruses that bind to integrins. For example, binding of foot-and-mouth disease virus and coxsackievirus A9 is reduced by 74 and 69%, respectively, by polyclonal antisera to the  $\alpha_3\beta_3/\beta_5$  integrin (2). Approximately 10% of the observed binding of VLPs to both HaCaT and CV-1 cells in our assay is trypsin insensitive and nonsaturable and may represent non-specific binding of damaged or denatured VLPs. However, this possible artifact would be insufficient to account for the observed lack of quantitative binding inhibition, as we observed an average of 63% inhibition of VLP binding to CV-1 and HaCaT cells. This partial inhibition may be a property of the GoH3 antibody, as this antibody inhibited  $\alpha_6$  binding to matrigel and laminin by 49 to 53% for primary human epidermal keratinocytes (8) and by 70% for the choriocarcinoma cell line JAR (11). Alternatively, there may be other receptors for VLPs on epithelial cells, and we are pursuing further experiments to address this question, as our studies revealed at least two other cell surface proteins, of 87 and 35 kDa, which can associate with VLPs. The anti- $\beta_4$  MAb was devoid of any blocking activity, but this does not completely rule out a role in VLP binding, as the  $\beta_4$  integrin has a large extracellular domain and the antibody used may bind to a noncritical region. The relative roles of  $\beta_4$  and  $\beta_1$  complexes in PV entry into cells might be inferred by studying the nature of PV infection in patients with hereditary deficiency of these proteins (22, 33).

The  $\alpha_6\beta_4$  integrin heterodimer is expressed exclusively in the basal layer of cells of stratified squamous epithelium (6, 8, 14, 29), which is presumably the only site of productive PV infection, and  $\alpha_6\beta_4$  integrin expression is rapidly turned off at the onset of cell differentiation (32). The  $\alpha_6\beta_4$  complex is an integral part of the hemidesmosome (29) and as a receptor for laminins 1, 2, 4, and 5 (31) is involved in the interaction of epithelial cells with the basement membrane. In support of the role of the  $\alpha_6\beta_4$  complex being a receptor for PVs, human laminin was able to block the binding of VLPs to HaCaT cells in a dose-dependent manner. The  $\alpha_6$  subunit is present over the entire surfaces of basal cells and some cells in the immediate suprabasal region, while the  $\beta_4$  subunit is localized to the basal pole of basal cells (29). By contrast,  $\beta_1$  integrin subunit expression is very weak on the basal aspect of basal epithelial cells but stronger on all other surfaces of these cells: it pairs with the  $\alpha_2$  and  $\alpha_3$  subunits in epithelial cells and is involved in cell-cell adhesion (29). Our data show that expression of  $\alpha_6$  integrin and binding of VLPs colocalize within the epithelium.

It is held that PVs infect basal epithelial cells and stimulate

their proliferation. Infection is believed to occur as a result of exposure of basal cells to virus particles after minor trauma to the epithelium. During wound healing, the  $\alpha_6\beta_4$  integrin becomes widely expressed over the entire surface of epithelial cells as they migrate to cover the wound site (18). During this process,  $\alpha_6\beta_4$  integrin is constitutively endocytosed and recycled, with a rate of endocytosis of 1 to 2% of surface molecules per min (5). Recycling of the receptor is believed to facilitate cell migration, during which the integrin (which is attached to the matrix) moves backwards on the cell as the cell advances. Internalization of viruses together with integrins occurs with another nonenveloped virus, adenovirus, which binds to  $\alpha_3\beta_3/\alpha_3\beta_5$  (36). We can therefore propose a model for PV infection in which virus particles bind to the  $\alpha_6\beta_4$  integrin during wound healing and are endocytosed during cell migration.  $\alpha_6\beta_4$  integrin is believed to be attached to the microfilament network via the hemidesmosome (10, 17, 31), and this may provide an explanation for the observation that cytochalasin B inhibits PV uptake in CV-1 cells (37), suggesting that PV particles endocytosed together with  $\alpha_6\beta_4$  integrin may be transported to the cell nucleus via a microfilament-dependent pathway.  $\alpha_6\beta_4$  integrin is also a signal transduction receptor (20, 23), and the possibility that binding of PV to its receptor is able to induce a state of altered viral permissiveness in the infected cell is therefore raised.

Other viruses also enter cells after binding to integrins. Echoviruses 1 and 8 bind to the integrin  $\alpha_2\beta_2$  (VLA-2) (1), the  $\alpha_3\beta_3$  integrin functions as a receptor for both coxsackievirus A9 (27) and foot-and-mouth disease virus (2), and the  $\alpha_3\beta_3/\beta_5$  integrins function as a secondary receptor for adenovirus (36). The  $\alpha_3\beta_3$  and  $\alpha_3\beta_3/\beta_5$  integrins recognize substrates that contain RGD motifs, and peptides containing this sequence effectively block viral binding. However, RGD-containing peptides do not affect PV VLP binding, ruling out these integrins as receptors.

Identification of the  $\alpha_6$  integrin as a component of the cellular receptor responsible for saturable binding of PVs to epithelial cells should allow studies of the mechanism of PV particle uptake into cells, which may clarify whether the tropism of PV for epithelial cells is due to the tissue specificity of the receptor or uptake mechanisms. Targeting PVs to other cell types through expression of the  $\alpha_6$  molecule will facilitate such studies. Identification of a receptor for PV particles should lead to identification of residues on the PV particle that are critical for PV binding and uptake and may allow a rational approach to design of drugs to inhibit the process of PV infection.

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# Protein Identification and Characterization by Mass Spectrometry

UNIT 10.22

Many aspects of science are driven by technological advances. The pace of molecular biology research continues to increase through such advances, many of which are described in *Current Protocols in Molecular Biology*. However, in the last decade significant technological advances in many aspects of protein chemistry have been realized. Most of these advances have been driven by new ionization capabilities of mass spectrometers, making the analysis of biological materials feasible, and most importantly, by a wider range of scientists. Together with the massive increase in the amount of nucleotide sequence data that is publicly available, this has led to the development of new methods for protein identification using mass spectrometry-derived data (reviewed in Patterson and Aebersold, 1995; and Pennington et al., 1997). In addition, protein identification by mass spectrometry-based methods is being adapted for massively parallel analysis on the one hand or very rapid serial analysis on the other, thereby resembling some of the extremely powerful molecular biological approaches currently available.

As we enter a post-genome era for many organisms, including ultimately *Homo sapiens*, these technologies will be applied to high-throughput approaches as well as in more traditional settings (Patterson, 1997). Such approaches will facilitate analyses of many of the proteins expressed by a given tissue or cell, now being referred to as proteome analysis. The term proteome refers to the *protein* complement expressed by a *genome* (Wilkins et al., 1996) and is often applied to two-dimensional gel electrophoresis-separated proteins and their subsequent analysis (James, 1997; Pennington et al., 1997). With so much nucleotide sequence information at hand in the form of full-length cloned genes and expressed sequence tags (ESTs) for a few species, the need for traditional de novo amino acid sequencing for these genetically well-characterized species is rapidly diminishing. A new era of protein identification by correlation of primary structural data with these databases has begun.

This overview will describe some of the new technologies that can be employed to facilitate rapid identification and characterization of proteins, including the use of correlative approaches for protein identification, rapid post-

translational modification analysis, identification of components in complex mixtures, and direct mass analysis of gel-separated proteins. Other fields of protein chemistry that have been impacted by the advances in mass spectrometry include analysis of protein:protein interactions of both purified proteins and ligands in solution/gas phase (Chapter 20; Robinson et al., 1994; Loo, 1997), and direct screening of specific analytes in natural fluids through the use of affinity surfaces (Nelson et al., 1995). Determination of domain structure through accurate mass analysis after limited proteolysis has also been used as an adjunct to X-ray crystallographic studies, including designing molecules of appropriate size for crystallization (Cohen, 1996). However, as it is not possible within this overview to cite all of the relevant literature, only a limited number of examples of each approach will be given, and where relevant, more extensive reviews will be cited.

The mass spectrometric methods that will be referred to in this overview include matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) and electrospray ionization mass spectrometry (ESI-MS). This overview will not include a detailed description of the technical side of mass spectrometry, as this is well covered in UNIT 10.21, and a discussion of its use for analysis of biological molecules appears in Chapter 16 of *Current Protocols in Protein Science* (Coligan et al., 1997). However, a brief description of the two types of mass spectrometers utilized in the studies mentioned in this overview follows.

Mass spectrometers can be thought of simply as having two main components, an ionization source and a mass analyzer. Charged molecules are generated by an ionization source, e.g., MALDI or ESI, into the gas phase of a mass analyzer which is held under vacuum. MALDI sources are usually coupled with time-of-flight (TOF) mass analyzers. In MALDI-MS, the sample (analyte) is cocrystallized in a matrix that absorbs at the wavelength of the laser—either an ultraviolet (UV) laser at 337 nm, or an infrared (IR) laser at 2.94  $\mu\text{m}$ . Ionization occurs in a static electric accelerating field that is generated by holding the sample at high potential (e.g., 20 to 30 kV) with respect to a closely spaced grounded electrode. The accelerated analyte ions pass through an orifice

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in the grounded electrode (referred to as continuous extraction) into a field-free drift region of 50 to 300 cm, and signals are recorded when they strike a detector at the end of the flight tube. All of the ions are accelerated by the same potential difference, hence their velocity is proportional to their mass over charge ( $m/z$ ), i.e., smaller ions arrive at the detector sooner than larger ions, and all of the ions generated in each ionization event are measured. Analytes of known mass can be used to calibrate the instrument. Depending upon the matrix used to cocrystallize the sample, the analytes are usually singly charged, i.e., only one proton has been added to the analyte. Mass over charge ratios of up to ~500,000 have been measured with MALDI-MS. In addition to continuous extraction, another form of analyte extraction from the MALDI probe has recently been introduced onto commercial MALDI-MS instruments and is referred to as delayed extraction (time-lag focusing; see UNIT 10.21). This allows the extracted ions time to focus prior to an acceleration of the gas-phase ions into the field-free drift tube resulting in improved sensitivity and increased mass accuracy by reducing the spread of flight times of ions of the same mass (e.g., due to surface nonhomogeneity; Vestal et al., 1995).

In ESI-MS, the analytes are introduced into the gas phase by passing them through a fine needle at high potential at atmospheric pressure (e.g., +5 kV, for positive-ion spectra) resulting in the formation of a fine spray of highly-charged droplets. This spray is directed across the small inlet orifice (held at a low potential, e.g., +50 V) to the evacuated quadrupole mass analyzer. Between the spray needle and the orifice, the droplets are desolvated by directed flows of dry inert gas and/or heat. These dry ions can now be mass analyzed in the quadrupole device. Due to this process, most analytes entering the mass analyzer are multiply charged, i.e., more than one proton has been added to the analyte during the ionization process; peptides, for example, are often only doubly (mass of peptide + 2 protons divided by 2 =  $m/z$  value) or triply charged, but proteins can carry many protons. Quadrupole mass analyzers are scanning instruments, unlike the TOF mass analyzer, where the masses of all of the analytes are measured following each ionization event. At any one point in time, only ions of one particular  $m/z$  value are passed through the quadrupoles; all other ions are lost for further analysis. A detector counts the number of ions transmitted at each specific  $m/z$  value,

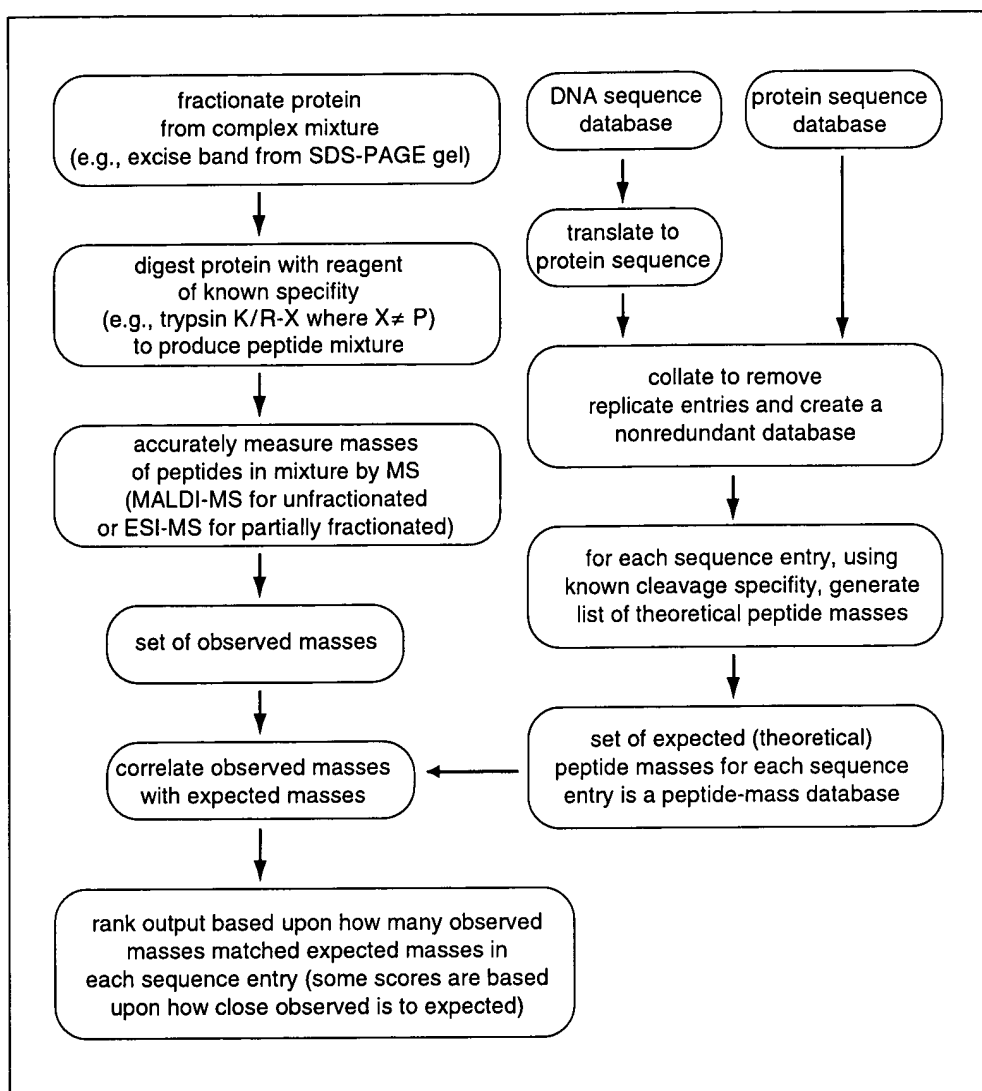
and by scanning a mass range, a spectrum of  $m/z$  versus abundance (a mass spectrum) can be plotted. The mass range of quadrupole instruments is limited compared with TOF analyzers, usually up to 2000 to 4000  $m/z$ , but as stated above, analytes ionized by ESI are multiply charged, and hence their  $m/z$  values are measurable in these instruments even when their masses are considerably lower than 2000 u (atomic mass units, equivalent to Da). In addition, in some mass spectrometers designed for that purpose, intact molecules (peptides usually) can be caused to break apart (i.e., fragment in a sequence-specific manner), and the masses of the resulting pieces can be measured (see Protein Identification Using Mass Spectrometry-Based Correlative Approaches and UNIT 10.21). For instruments that are appropriately equipped, this is referred to as collisional-induced dissociation (CID), or tandem MS (MS/MS) for ESI-MS, and as post-source decay (PSD) for MALDI-MS (see UNIT 10.21). The fragment ions produced are predominantly formed via fragmentation through the peptide bonds. The mass difference between fragment ions of the same series (either N- or C-terminal) is the residue mass of the amino acid at that position. Thus, the fragment-ion spectrum can be correlated with the peptide amino acid sequence.

Most simply put, mass spectrometers are very accurate balances providing an absolute physical measurement of analytes including proteins, peptides, and following fragmentation in the gas phase, sequence-specific fragment-ion spectra of peptides selected in the mass spectrometer.

## PROTEIN IDENTIFICATION USING MS-BASED CORRELATIVE APPROACHES

### Peptide-Mass Searching

Definitive protein identification for many years has been achieved by generating an amino acid sequence through chemical degradation of the N-terminus of the isolated protein (Edman degradation), or peptides derived from it (UNIT 10.19). Proteins have also been identified by immunodetection with antibodies recognizing the protein of interest (UNIT 10.8). A few years after it was practical to accurately measure the mass of peptides generated from gel-separated proteins, several groups published variations on the following theme as a means to identify proteins already resident in a sequence database: use an algorithm to match a set of peptide



**Figure 10.22.1** Protein identification using peptide-mass data generated by accurate mass measurement by either ESI-MS or MALDI-MS

masses generated in a specific manner (e.g., by enzymatic digestion with trypsin) from the protein of interest, with theoretical peptide masses calculated from each sequence entry in the database using the same cleavage specificity as the reagent in the experiment. Then derive a ranking (or score) to provide a measure of the fit between the observed and expected peptide masses (see Fig. 10.22.1; reviewed in Cottrell, 1994; Patterson, 1994; Patterson and Aebersold, 1995). Many of these original groups and others have placed their search programs on the World Wide Web for public access, and many include examples for their use (see Table 10.22.1).

The obvious caveat to such an analysis is that the protein to be identified already exists within a sequence database, either translated

nucleotide or protein sequence database. Therefore, this approach is ideal when the protein to be identified is derived from an organism whose genome is sequenced or at least whose major proteins are sequenced. Of course, such searches are not suited to translations of expressed sequence tag (EST) databases which consist of only limited coding regions and therefore, may not contain sufficient information for a match. Peptide-mass data is most rapidly obtained by MALDI-MS analysis of an aliquot of a protein digest. However, peptide-mass data can also be obtained from liquid chromatography (LC)-MS analysis of a protein digest.

It is rare that all of the input peptide masses match with the highest ranking sequence entry, and it is important to determine the origin of

**Table 10.22.1** Sites Providing Online Protein Identification using Mass Spectrometry–Derived Data

Resource	WWW Uniform Resource Locator ( <a href="http://...">http://...</a> )	Features and comments
Rockefeller University New York, N.Y.	<a href="http://prowl.rockefeller.edu/PROWL/prot-id-main.html">prowl.rockefeller.edu/PROWL/prot-id-main.html</a>	Both peptide-mass and fragment-ion search programs
ETH-Zurich Zurich, Switzerland	<a href="http://cbrg.inf.ethz.ch/subsection3_1_3.html">cbrg.inf.ethz.ch/subsection3_1_3.html</a>	Peptide-mass search program
University of California San Francisco, Calif.	<a href="http://prospector.ucsf.edu">prospector.ucsf.edu</a>	Both peptide-mass (MS-Fit) and fragment-ion (MS-Tag) search programs
Max Delbrück Centre Berlin, Germany	<a href="http://www.mdc-berlin.de/~emu/peptide_mass.html">www.mdc-berlin.de/~emu/peptide_mass.html</a>	Peptide-mass search program
EMBL Heidelberg, Germany	<a href="http://www.mann.embl-heidelberg.de/Services/PeptideSearch/PeptideSearchIntro.html">www.mann.embl-heidelberg.de/Services/PeptideSearch/PeptideSearchIntro.html</a>	Both peptide-mass and fragment-ion search programs
SEQNET Daresbury, UK	<a href="http://www.dl.ac.uk/SEQNET/mowse.html">www.dl.ac.uk/SEQNET/mowse.html</a>	Both peptide-mass and fragment-ion search programs

these masses (Patterson and Aebersold, 1995). The following should be considered as reasons for additional masses. (1) The correct protein was identified in the search, but the masses are the result of posttranslational or artifactual modification or posttranslational processing. Plausible modifications may be taken into consideration but should only be considered tentative unless confirmed experimentally. (2) The correct protein was identified, but some peptides were derived by nonspecific proteolysis or cleavage by a contaminating protease. This theory can be tested by determining whether the protein candidate can produce the peptide masses without any assumptions as to the cleavage specificity. (3) The correct protein was identified, but the “pure” protein was contaminated with one or more additional proteins. If there are enough additional masses, a separate peptide-mass search using these masses can be performed to attempt to identify the contaminant(s). (4) A sequence homologue, or processing variant, from either the same or a different species was identified. Some search programs allow species-specific searches to be performed, and that could eliminate this possibility; however, if confirmatory data is obtained, matches to proteins from other species can be useful, especially for scientists working with organisms whose genomes are relatively poorly characterized. (5) The match was a false positive! This potentially disastrous result is difficult to verify or disprove, particularly if the highest ranked protein did not have a high score; sometimes confidence in the highest-ranked score can be gained from the difference

between it and the second highest and subsequent scores; the protein may also not yet reside in the database and it may be truly novel.

Implicit to the theory of peptide-mass searching is the accurate measurement of peptide masses. Therefore, the greater the accuracy, the greater the confidence of the assignment. In addition, the reagent used to generate the peptides should also be one that shows a high degree of specificity. This latter point is limited to the specificity of enzymes and chemical cleavage reagents. Trypsin is the most commonly used reagent for peptide-mass searching analyses, but it can also cleave at sites other than C-terminal to Lys or Arg, if not followed by a Pro, and as with most enzymes it may not cleave a substrate to completion (i.e., missed cleavage sites). The number of missed cleavage sites is often a variable for peptide-mass search routines, but spurious cleavage sites for trypsin or other enzymes cannot be predicted. Endoproteinase LysC is another good enzyme used in such analysis, but it can also miss cleavage sites and cleave nonspecifically; this enzyme has the advantage that there are few if any autolysis products. Thus, there is little that can be done to improve the specificity of the reagents to cleave isolated proteins, but more accurate mass measurement is in large part dependent upon the type of mass spectrometer employed.

Most peptide-mass analyses are performed on MALDI-mass spectrometers due to the tolerance of the MALDI process to sample buffers and its rapid and simple analysis. This has increased further in the last couple of years

following implementation of delayed extraction which dramatically improves the mass accuracy obtainable on an ever-increasing range of commercial instruments (Vestal et al., 1995). The ability to measure masses at 5 ppm allows assignment of monoisotopic masses and the use of very tight tolerances in peptide-mass searches, thereby limiting the number of potential matches (Jensen et al., 1996a; Takach et al., 1997). Even though increasing the mass accuracy can't affect any of the situations mentioned above, there is less chance of a spurious match.

In addition to more accurately measuring the mass, a number of orthogonal methods can be performed on a separate aliquot of the peptide digest (or sometimes for MALDI-MS on the probe after the original data has been obtained) to provide information on the amino acid composition of each peptide, which can be used as an additional piece of information for the peptide-mass search algorithms. This is achieved through one of the following approaches: site-specific chemical modification [e.g., methyl esterification, which adds +14 u for each acidic residue—Asp, Glu, or C-terminus—in the peptide (Pappin et al., 1995) or iodination, which adds +126 u for each tyrosine (Craig et al., 1995)]; determination of partial amino acid composition of the peptide (hydrogen/deuterium exchange in which each amino acid can exchange a defined number of its hydrogens for the heavier deuterium, and the total mass increase reflects the peptide composition from 0 to 5 u per residue; James et al., 1994); one-step Edman degradation to determine the N-terminal amino acid of each peptide in the mixture (Jensen et al., 1996b); or an additional enzymatic digestion [subdigestion (Pappin et al., 1995), or parallel digestion (James et al., 1994)].

These approaches have proven quite successful for a number of investigators (reviewed in Patterson and Aebersold, 1995; Jungblut et al., 1996; Lamond and Mann, 1997). Most importantly, the data can be obtained very rapidly when using MALDI-MS of peptide mixtures, and some groups have implemented automated approaches so that large numbers of proteins can be analyzed in an unattended mode (see below). In one report, a peptide-mass search result provided sufficient information to be able to clone a homologous gene because the sequence of the matched peptides could be used to generate primers (Salmeron et al., 1996).

### Peptide-Sequence Tags, Uninterpreted Fragment-Ion Searching, and de Novo Sequencing by MS/MS

The most discriminating criteria used to identify a protein is contiguous amino acid sequence information. The amino acid sequence information can be obtained either by chemical sequencing (automated Edman degradation) or by generating sequence-specific fragment ions from individual peptides in the gas-phase of a mass spectrometer (see UNIT 10.21 for detailed description of fragmentation; see Figure 10.21.1 for fragmentation nomenclature). If sufficient contiguous amino acid sequence is obtained from either method, it is unnecessary to generate additional data to confirm the identification of the protein because standard string-based search protocols (e.g., FASTA; see UNIT 19.3) can be employed. However, this is not always possible, and use of accurate mass measurement in conjunction with partial amino acid sequence information can be a very powerful approach.

Measurement of the masses of fractionated peptides (usually by reversed-phase-HPLC) prior to automated Edman sequencing provides the researcher with a number of options in addition to the amino acid sequence obtained. (1) Mass information can make it possible to determine more accurately the number of sequencing cycles required, thus minimizing reagent expense and sequencer time. (2) The peptide mass can confirm the determined peptide sequence and whether the sequence is complete (the sum of the masses of the identified amino acids should equal the mass of the peptide). (3) The mass analysis usually indicates the purity of the sample, thereby avoiding sequencing mixtures (although this is not always the case). (4) Given that proteins are cleaved with rather high enzyme-to-substrate ratios, knowledge of the mass of the peptide to be sequenced can avoid sequencing of an enzyme autolysis product. (5) An amino acid sequence can sometimes be completed if only a couple of amino acid residues have not been assignable, i.e., by summing the masses of the assigned residues and determining which residues could account for the mass difference between that and the mass of the intact peptide (Tempst et al., 1995). Thus, simple measurement of the mass of a peptide is an extremely useful exercise. Another scenario, often faced when sequencing low levels of peptides, is that only a few amino acid residues of a sequence are obtained, insufficient to identify the protein or to construct an oligonucleotide primer. If the

mass of the peptide has been determined, this limited amino acid sequence data may still be sufficient for further use (e.g., Patterson et al., 1996).

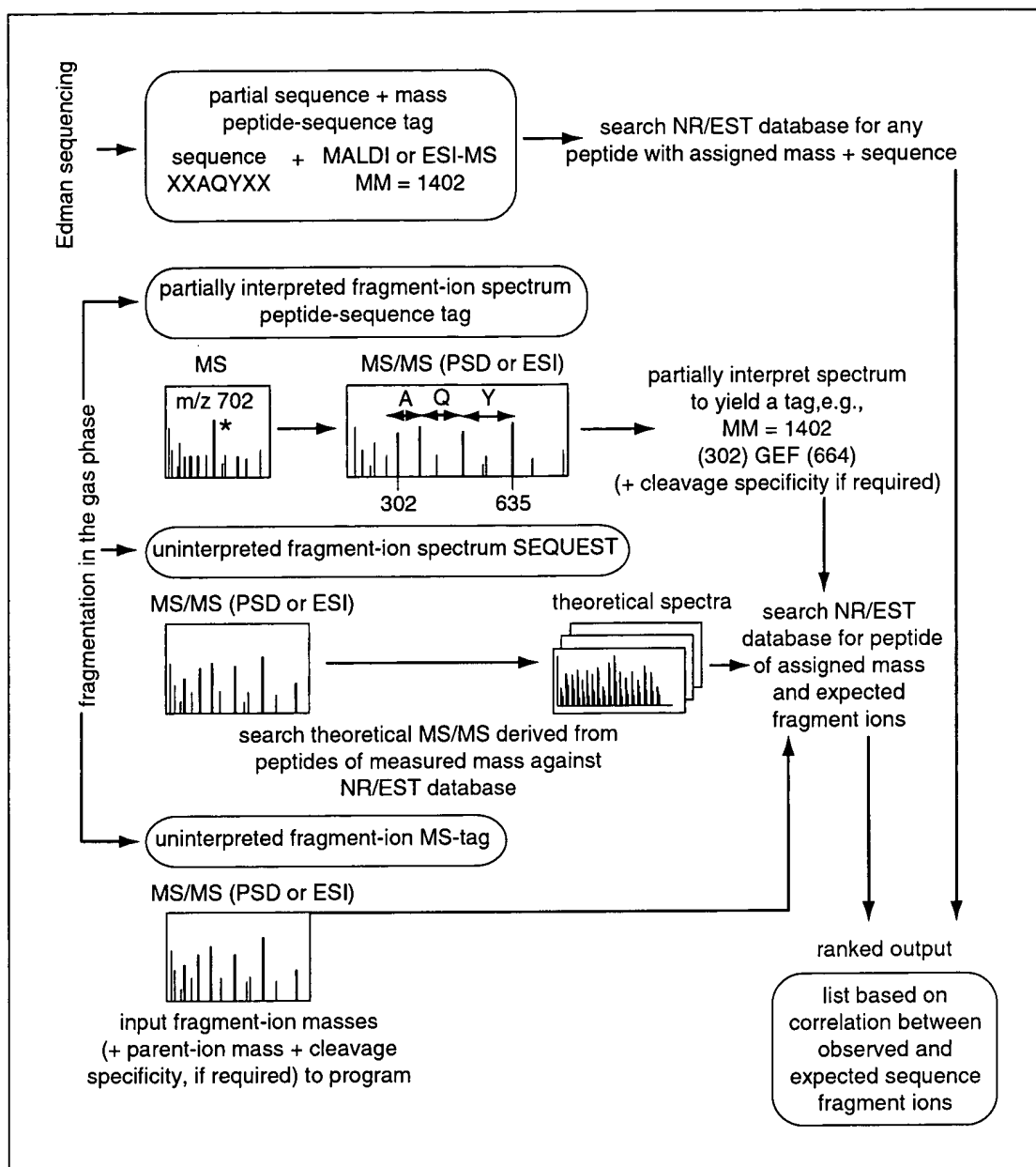
The partial amino acid sequence, which does not need to be contiguous, and the mass of the peptide can be used to construct a "peptide-sequence tag." This term was coined by Mann and Wilm (1994) to reflect the power of this relatively small amount of information to identify the protein from which specific peptides were obtained, much the same way short stretches of nucleotide sequence as expressed sequence tags (ESTs) have proven so useful. By using the partial amino acid sequence, the cleavage specificity of the enzyme or other reagent used to generate the peptide, and the peptide mass, the peptide-sequence tag can be used to search amino acid sequence databases (including translations of EST databases). Mann and Wilm (1994) also described a method by which partial interpretation of sequence-specific MS/MS fragment-ion spectra (see UNIT 10.21) combined with the mass of the peptide from which the spectrum was obtained can be used to construct a peptide-sequence tag (see Fig. 10.22.2). In this case a portion of the fragment-ion spectrum is interpreted to determine whether an ion series (either *b* or *y*; see Fig. 10.21.1 in UNIT 10.21) can be established, i.e., a series of fragment ions whose mass differences correspond to known amino acid residues. The peptide-sequence tag is composed of three regions: the mass of the first ion in the series which provides the mass of the peptide fragment from an end of the peptide to the uninterpreted region; the interpreted amino acid sequence for anywhere from one to many residues; and the mass of the last ion in the series (the difference between this and the mass of the intact peptide is the mass of the uninterpreted region between the sequence and the other end of the peptide; Mann and Wilm, 1994; see Fig. 10.22.2). If the cleavage specificity of the enzyme is used, two additional pieces of information are then included in the search—the N-terminal and C-terminal specificity. These can also be left out if the search is conducted in error-tolerant mode which allows for either database sequence errors or posttranslational modifications.

Yates and colleagues have developed identification algorithms, referred to as SEQUEST, that are based upon automated interpretation of MS/MS fragment-ion spectra in a manner compatible with direct searching of sequence databases (Eng et al., 1994). In this approach, the

program first generates a list of theoretical peptide masses for each sequence entry, using either enzyme cleavage specificity rules or the sum of contiguous amino acid residues, and calculates which of these match the measured mass of the peptide ion (within a stated tolerance), thereby generating a list of candidate peptides. In the second step, the program then calculates the fragment ions expected for each of the candidate peptides if they were fragmented under the experimental conditions employed to generate a predicted spectrum; then it compares the experimentally determined MS/MS spectrum with the predicted spectra using cluster-analysis algorithms. Each comparison receives a score, and the highest-scoring peptide sequences are then reported. If a significant score is achieved, the protein is identified based on the peptide MS/MS spectrum without any explicit determination of the peptide sequence (see Fig. 10.22.2).

The advantages of such an approach include the following (Patterson and Aebersold, 1995). (1) Proteins can be effectively identified in complex mixtures because each peptide that generates an MS/MS spectrum provides data for an independent database search. (2) When applied to analysis of a digest from a single protein, the results are inherently autoconfirmatory, and with the same protein as a top-ranked candidate for a number of peptides, identification is absolutely confirmed. (3) The approach allows for easy automation when ions for fragmentation are selected in a data-dependent fashion, and the MS/MS results are automatically analyzed (Yates et al., 1995). (4) The method is able to be adapted to find peptides carrying specific posttranslational modifications (the program can be set to anticipate a modification of a specific mass on specific residues) as well as to identify the protein from which the peptide originated (Yates et al., 1995). A number of other investigators have developed algorithms for uninterpreted fragment-ion searches based upon matching of observed and expected fragment-ion masses, and a list of sites on the Internet that provide these search capabilities is provided in Table 10.22.1. One of these referred to as MS-Tag (Matsui et al., 1997), developed at the University of California, San Francisco, uses an algorithm to match the observed fragment-ions with a user-selected list of specific ion series; this is also shown in Figure 10.22.2.

Manual or computer-assisted interpretation of MS/MS spectra can be very difficult in many instances because it is impossible to determine

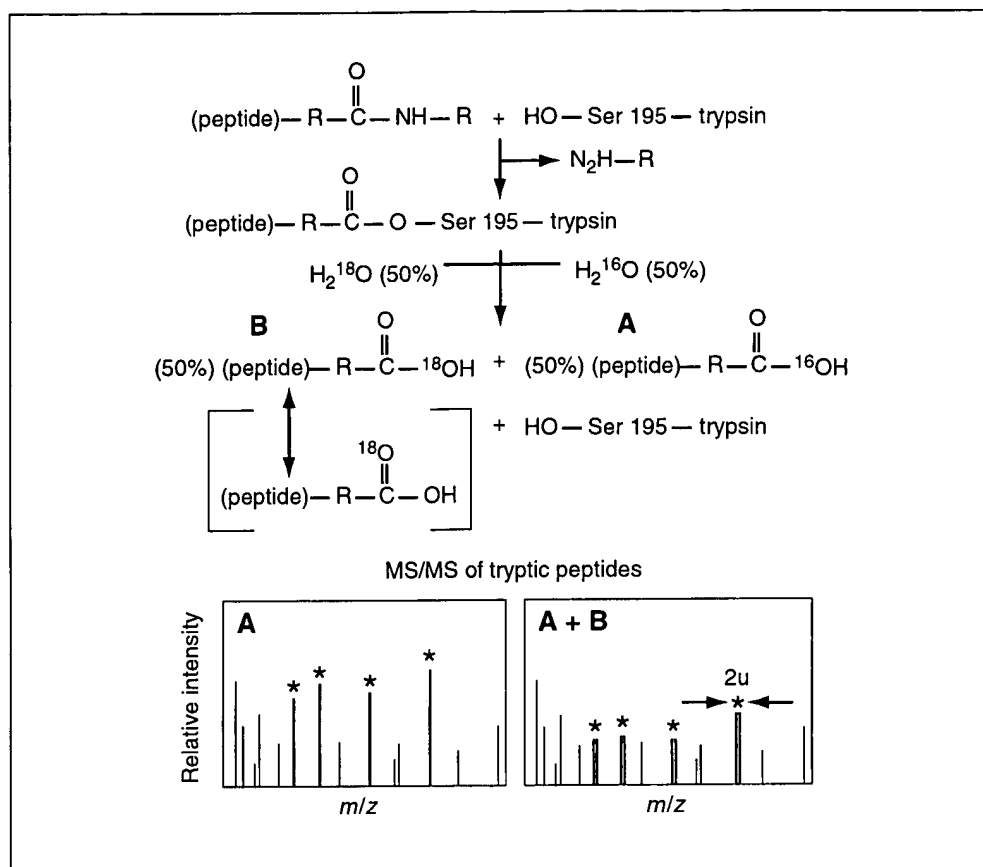


**Figure 10.22.2** Protein identification using fragment-ion data, interpreted or not. For a peptide of interest the mass is accurately measured by MS and sequence information is generated by either Edman sequencing (if an incomplete sequence is obtained), fragmentation in the gas phase by ESI-MS/MS, or PSD-MALDI-MS to yield a fragment-ion spectrum.

which series-specific ions belong to N-terminal- (*b*) or C-terminal- (*y*) derived peptides; hence, the great utility of the methods described above for peptide sequence tagging and uninterpreted fragment-ion searching is for searching protein sequence and translated nucleotide sequence databases. However, for these approaches to be successful, the sequence that matches the peptide under analysis has to reside in the database. ESTs constitute only a portion of the sequence of many but not all expressed genes. Many widely used experimental organ-

isms are poorly represented in the sequence databases, and although conserved regions between proteins may be able to be matched (Cordwell et al., 1997), more often than not an accurate extended amino acid sequence is required so that oligonucleotide primers can be made to obtain the gene of interest from an appropriate library via the polymerase chain reaction (PCR; UNIT 22.1).

Mass spectrometry-based approaches are the methods of choice for generating sequence-based information from very low levels of pro-



**Figure 10.22.3** A scheme for de novo sequencing using  $\text{H}_2^{18}\text{O}$ . Tryptic digestion in the presence of 50%  $\text{H}_2^{18}\text{O}$  facilitates de novo sequencing by MS/MS by generating two sets of  $y$  series ions (\*) that differ in mass by 2 u (compare MS/MS of peptide A with the mixture of peptides A and B).

tein. Therefore, for proteins from species with limited sequence information, de novo sequencing from MS/MS spectra is important. This can be accomplished in two ways; (1) by generating two MS/MS spectra of the peptide with and without methyl esterification, and (2) by conducting tryptic digestion in the presence of 50% (v/v)  $\text{H}_2^{18}\text{O}$  (see Fig. 10.22.3). In the first method, a MS/MS spectrum is obtained after all of the carboxyl groups have been modified by esterification with a methyl group thereby increasing the mass of the peptide by 14 u for each carboxyl group present. This method is most useful when the peptide of interest does not contain any acidic residues, and it is of least use when either multiple acidic residues are present or the one acidic residue is located on the N-terminus. If there are no acidic residues, then comparison of the MS/MS spectra with and without esterification will reveal that all  $y$ -series ions are 14 u heavier in the methyl-esterified spectrum and that all of the other ions are unaffected. If there are acidic residues in the peptide, the comparison becomes more complicated.

A more rapid approach for de novo sequence interpretation is afforded by conducting the original tryptic or LysC digestion in the presence of 50% (v/v)  $\text{H}_2^{18}\text{O}$ /50% (v/v)  $\text{H}_2^{16}\text{O}$  (Schnolzer et al., 1996). This results in all of the newly generated peptides but not the C-terminal peptide being present as doublets differing in mass by 2 u; it also reduces the intensity of the total signal for the intact species by half. This occurs because trypsin cleaves the peptide bond after lysine or arginine via the activated OH group of serine-195 (of trypsin), which forms a covalent ester bond with the carboxyl group of the lysine or arginine residue (the newly formed N-terminus is released). This intermediate is then cleaved by attack of an activated water molecule, leading to release of the peptide fragment, whose carboxyl group contains one oxygen from solvent water (Schnolzer et al., 1996). If the solvent water is 50%  $\text{H}_2^{18}\text{O}$  then half of the peptides will have a mass 2 u greater than those that incorporate  $^{16}\text{O}$  from normal water. Therefore, when these peptides are fragmented, all of the C-terminal-derived peptides ( $y$ -series ions) will exist as



doublets allowing identification of one ion series. In many cases complete interpretation of the MS/MS spectrum in a single experiment is possible, especially if high-resolution MS instruments are employed (Shevchenko et al., 1997).

## **DIRECT MS ANALYSIS OF GEL-SEPARATED PROTEINS AND OTHER MULTI-DIMENSIONAL APPROACHES FOR PROTEIN SEPARATION AND MASS MEASUREMENT**

### **MS and MS/MS Analysis of Proteins and Peptides Following Gel Electrophoresis**

The last separation step prior to protein identification and/or characterization often involves electrophoretic separation. As described, mass spectrometry has provided many powerful approaches to protein identification. Methods compatible with mass spectrometry analysis are most often applied to gel-separated proteins and involve generation of peptides by digestion of the protein in the gel or following transfer to a membrane (reviewed in Patterson, 1994; Patterson and Aebersold, 1995). The peptides are then extracted and analyzed as a mixture or alone following separation. One way to speed up this process and potentially avoid losses due to multiple handling steps is to perform all of the mass spectrometry analyses either directly out of the gel or following transfer to a membrane. As MALDI-MS is compatible with solid-phase methods, it is the analytical method of choice. It also provides an opportunity to measure the mass of the intact molecule prior to digestion and analysis of the resulting peptides.

Transfer of the intact proteins to a membrane has always been a preferred step because it allows many potential ionization-suppressing contaminants to be washed away and matrix to be introduced easily. Therefore, it was considered appropriate to transfer proteins to a membrane prior to any attempt to ionize them intact in a mass spectrometer. A number of different membranes have been evaluated for subsequent MALDI-MS analysis of intact proteins using either ultraviolet (UV) or infrared (IR) lasers (reviewed in Vestling and Fenselau, 1995). For proteins or peptides to be ionized directly from a membrane, they first have to be extracted into a solvent that contains matrix, so the protein can cocrystallize with matrix. Therefore, the stronger the interactions between the protein

and the membrane, the less protein will be available for ionization. The choice of solvent for the matrix is also important as solvents with high organic content can eliminate the spatial resolution of the electrophoretically separated proteins due to diffusion (Vestling and Fenselau, 1994a; Loo et al., 1996). In addition, when proteins are electroblotted onto membrane, they often penetrate the surface of the membrane and may not be accessible to the laser (especially the short-wave-length UV lasers generally employed in commercial MALDI-MS instruments). Data suggests that UV lasers are not as efficient at ion extraction as IR lasers are when the analyte is embedded in a membrane following electroblotting, although the effect is less pronounced when the protein is spotted and not electroblotted onto the membrane (Strupat et al., 1994; Schreiner et al., 1996). This was confirmed by the rather large quantities of protein required for detection on the membrane compared to that which could be analyzed by enzymatic digestion (Patterson, 1995; Schreiner et al., 1996). IR-MALDI-MS has been shown to be more efficient at extracting proteins from electroblotted membranes (Strupat et al., 1994), and it has recently been shown to be directly compatible with two-dimensional gel electrophoresis separations and blotting to PVDF (Eckerskorn et al., 1997). This latter study revealed that an area of a blotted membrane could be effectively converted into a mass contour plot that displayed significant microheterogeneity within individual two-dimensional gel electrophoresis-separated protein spots that were not resolved electrophoretically (Eckerskorn et al., 1997). Thus the power of two-dimensional gel electrophoresis combined with accurate mass measurement of intact proteins by IR-MALDI-MS will no doubt be of significant benefit to identification and characterization projects. Current limitations include the small area of the membrane that can be placed into the MALDI-MS, but more of an issue is the amount of data generated and its collation into a format for easy analysis. However, there is no reason to imagine that either of these problems is insurmountable.

Another important issue to be addressed is the further analysis of the protein after the intact mass has been determined. The intact mass is very useful for characterization purposes, but it will not lead to confident identification, which requires generation of peptides for peptide-mass searches and sequence-specific fragment ions from individual peptides for peptide-

sequence tag or uninterpreted fragment-ion searches (see Protein Identification Using Mass Spectrometry-Based Correlative Approaches). This has already been demonstrated, at relatively high picomole loads on the gel, by a number of investigators where chemical or enzymatic cleavages followed by direct UV-MALDI-MS of the resulting peptides (without elution) have been achieved, allowing peptide-mass searching to be used for protein identification (Vestling and Fenselau, 1994a,b). Fabris et al. (1995) also demonstrated that peptides generated by enzymatic cleavage on the membrane could be fragmented by post-source decay (PSD) UV-MALDI-MS. These latter two steps have been achieved for IR-MALDI-MS in a continuation of previously published work (Eckerskorn et al., 1997), according to reports at a recent meeting (F. Lottspeich, pers. comm.), where results were shown for measurement of the intact mass of the protein, peptide masses produced by enzymatic digestion, and PSD-MALDI-MS spectra of some individual peptides, all obtained from individual electrobotted proteins. Such analyses demonstrate the utility of the approach and lay the groundwork for its adaptation to larger scale "proteome" projects.

Originally it was also considered difficult for proteins and peptides to be extracted in an electric field from a gel-matrix, so membrane-based methods were used. However, the Andrews group has demonstrated in a series of proof-of-principle experiments that direct UV-MALDI-MS from certain types of gels is possible (Loo et al., 1996). By separating proteins in an isoelectric focusing (IEF) gel and subjecting this gel to delayed extraction-MALDI-MS after drying the gel and applying matrix, they were able to generate a virtual two-dimensional gel (Loo et al., 1996; see Fig. 10.22.4). The same considerations outlined above for membrane-based approaches apply to analyses of proteins out of the gel—e.g., sensitivity, visualization of the protein in a manner compatible with further analysis, matrix and solvent combinations to allow cocrystallization of the protein from the gel without loss of the spatial resolution, thin gels so that the protein concentration per gel volume ratio isn't too low, and methods compatible with peptide generation and subsequent sequence-specific fragmentation generation. This research was originally performed on a standard continuous-extraction UV-MALDI-MS, but with delayed-extraction UV-MALDI-MS significant improvements in mass accuracy were realized for the analysis of

intact ultrathin (0.35-mm) IEF gel-separated proteins, as well as cyanogen bromide digests of these same proteins (Loo et al., 1997). Sensitivities were in the low to subpicomole range, were most compatible with IEF gels, but were also successful, albeit at higher protein loads, with SDS-PAGE gels (Loo et al., 1997). It is yet to be demonstrated whether PSD-MALDI-MS can be performed on peptides generated in the gel, but as this is an ionization-dependent event, it would not be considered to be a problem.

Therefore, these new approaches for direct analysis of gel-separated proteins either from the gel or following transfer to a membrane show considerable promise for future automated protein identification and characterization protocols.

### Multidimensional Separations with MS as the Final Dimension

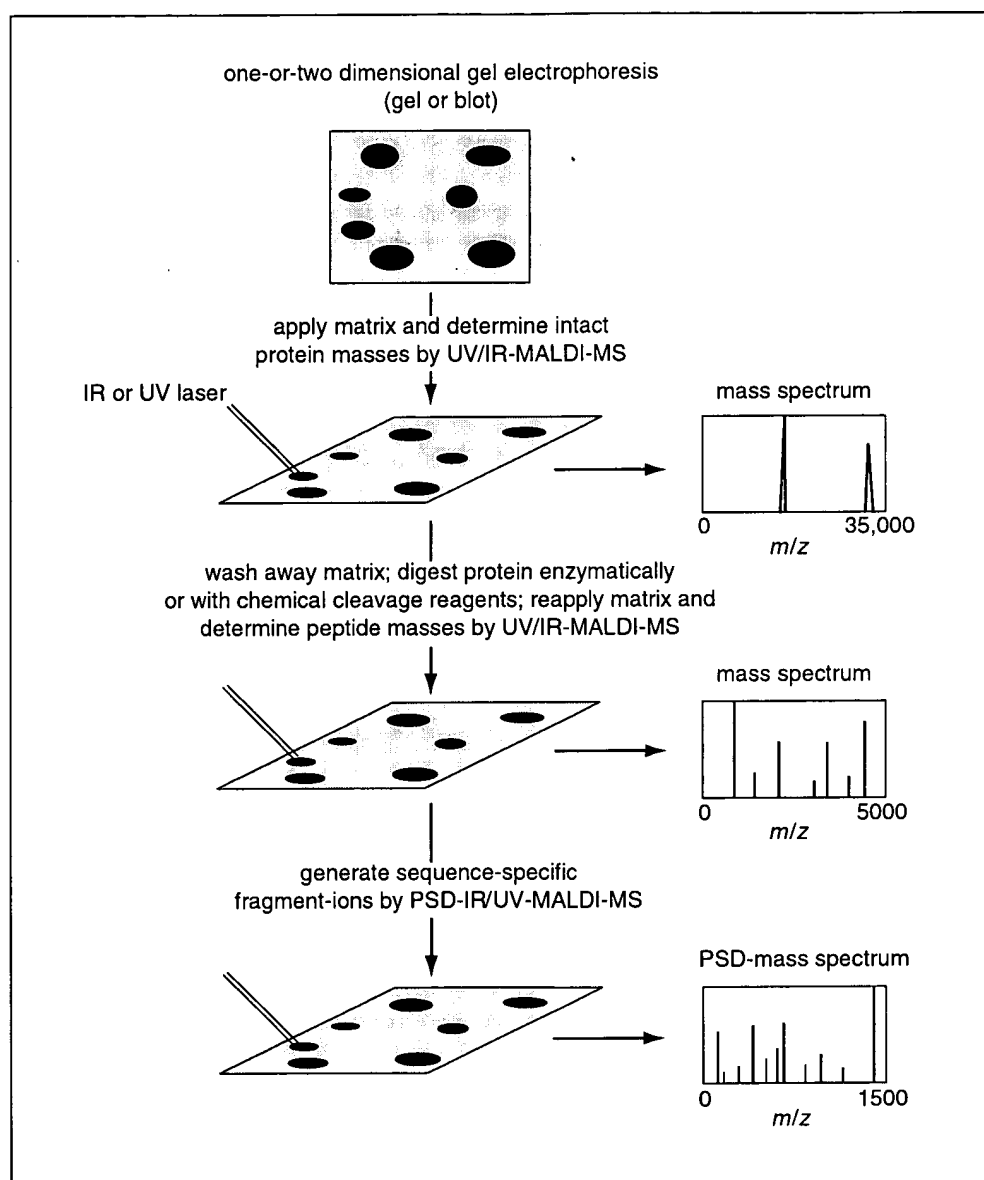
In classical two-dimensional gel electrophoresis the second dimension provides a mass estimate based upon comparison of SDS-denatured proteins with similarly treated standards. As described in the previous section, substitution of mass spectrometric detection for gel electrophoresis (or as an addition) provides a very accurate mass for each separated component that can be ionized. By employing a liquid-phase first dimension, introduction of the protein mixtures into ESI-mass spectrometers becomes possible by facilitating integration of a series of on-line separation devices (see Fig. 10.22.5). Two examples of such approaches are described for protein analysis, one using IEF coupled to ESI-MS, the other using two orthogonal steps of chromatography on-line with ESI-MS.

Tang et al. (1997) described a capillary IEF gel system interfaced to an ESI-MS for the analysis of total cellular proteins from *E. coli*. They were able to discern just over 100 proteins by this approach, which is comparable with a minigel two-dimensional gel electrophoresis approach where an equal number of proteins were observed by silver staining. Comparison with high-resolution two-dimensional gel electrophoresis would have yielded a dramatically different result, but as with other methods described in this unit, it provides a glimpse of potential future approaches for analysis of complex protein mixtures. Opiteck and coworkers (Opiteck et al., 1997a) utilized two types of chromatography, cation-exchange and reversed-phase, coupled to two separate sets of LC pumps under computer control to separate

proteins on-line for subsequent ESI-MS analysis. Storage loops were used to collect effluent from the cation exchanger for subsequent injection onto the RP-HPLC which was connected to a UV detector as well as an ESI-MS. A limited protein mixture as well as an *E. coli* lysate were separated using this approach in a 2-hr run time (Opiteck et al., 1997a).

Both of these approaches demonstrate the ability to separate and accurately mass analyze

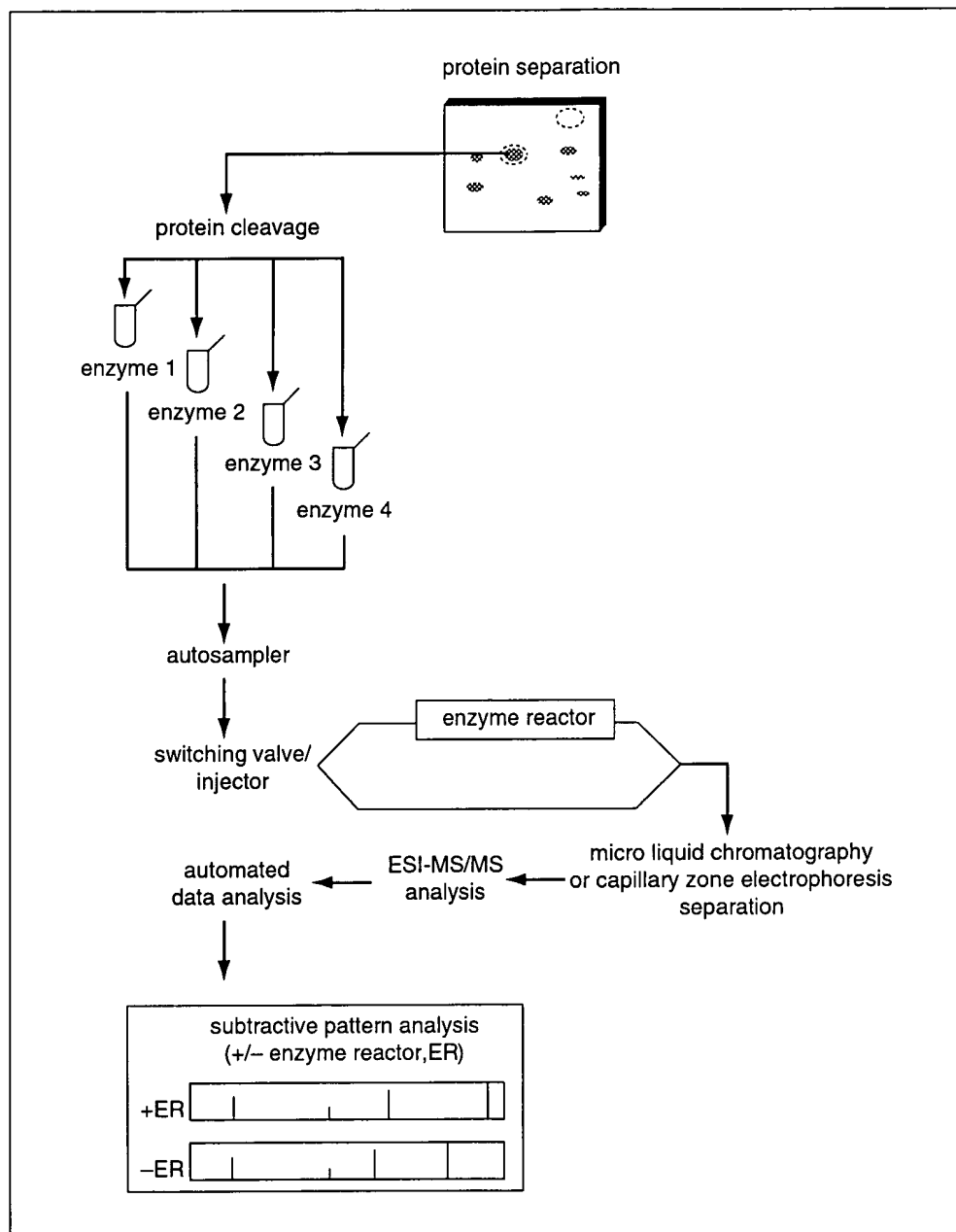
complex protein mixtures with some success. Before such approaches are competitive with two-dimensional gel electrophoresis, they will need to be able to detect proteins over a much larger range of concentrations and to demonstrate that the ionization intensity of any species is not affected by the concentration of a co-ionizing species, so the approach is truly as quantitative as is possible within limits for two-dimensional gel electrophoresis. Another draw-



**Figure 10.22.4** Direct mass measurement of gel-separated proteins. By inserting either dried thin gels or blotted membranes into a MALDI-MS with an ultraviolet (UV) or an infrared (IR) source, accurate mass measurements of the gel-separated proteins can be made. A "virtual" two-dimensional gel is the result of multiple MALDI-mass measurements of isoelectric (IEF) gel-separated proteins along the length of the IEF gel, thereby generating a mass contour plot of the IEF separation. Ultimately, sequential mass analyses (intact, following enzymatic/chemical cleavage, and sequence-specific fragmentation of individual peptides) will be able to be performed on the same protein spot/band as illustrated.

back of the approach is the inability (at this stage) to fragment the proteins from coeluting mixtures and determine the mass or sequence of the fragments, thereby allowing protein identification. However, it has been demonstrated that using a high-resolution mass spectrometer, CID fragmentation of intact proteins yielded short stretches of sequence-specific fragment ions that could be used in peptide-sequence-tag searches to identify the protein (Mortz et al.,

1996). This was applied to pure protein preparations but as specific ions from each protein were chosen, this approach may become suitable for application to the on-line protein-separation methods described above. With the significant advances that have been made in this field, it will be one to watch in the future.



**Figure 10.22.5** Schematic representation of an integrated system for determining posttranslational modifications. A range of enzymes can be employed to generate peptides covering the entire sequence of the protein of interest, and when a specific modification is being targeted, an enzyme reactor column can be placed on-line to remove the modification from an aliquot of the peptide digest (modified from figures supplied by Dr. R. Aebersold, University of Washington, Seattle, USA).

## RAPID POSTTRANSLATIONAL MODIFICATION ANALYSIS

Given that mass spectrometers are accurate balances, they are ideal instruments to identify posttranslational modifications in an unbiased manner without the use of radioactive precursors, e.g., one will only observe phosphorylation if radiolabeled phosphate is measured in an experiment. An ideal situation would be to be able to identify and characterize all of the posttranslational modifications present on a protein in a single experiment. To achieve this aim requires an approach to unambiguously characterize any and all posttranslational modifications that are stable to the mass spectrometric ionization process on an identified protein. This can be accomplished by mass measurement of (1) the intact protein to estimate the type and number of modifications, (2) peptides which represent all of the protein to localize the site of modifications, and (3) fragment ions of the modified peptide(s) to identify the site of modification. Many posttranslational modifications have been identified in an unbiased manner through the use of mass spectrometry analysis, and in many cases Edman sequencing, of recombinant proteins, often as a consequence of their overexpression (Tu et al., 1995 and references therein). However, fulfilling all of the above-mentioned criteria is a daunting task even when the protein has been obtained from recombinant sources and is therefore available in relatively high quantity. Characterization of *in vivo* protein modifications is the ultimate aim, and progress is being made towards this end. The applications of new protein technologies to this field are demonstrated through examples of analysis of phosphorylation.

The most reported posttranslational modification is that of phosphorylation due to its critical role in signaling pathways, predominantly through either the addition or removal of phosphate esters from the hydroxyamino acids serine, threonine, and tyrosine residues (Hunter, 1987; Charbonneau and Tonks, 1992). Phosphoramidates can also be formed with histidine, arginine, and lysine residues, and acylphosphates with aspartic and glutamic acids residues, but these are considered less frequent and therefore, their functions are less well understood. However, due to the labile nature of some of these modifications they may well be more frequent and important than currently assumed (Huang et al., 1991).

## Classical Approaches for Study of Protein Phosphorylation

Briefly, classical methods for the analysis of protein phosphorylation use two different approaches to add radiolabeled phosphate to proteins—metabolic labeling which involves equilibration of the cellular ATP pool with  $^{32}\text{PO}_4$  (see UNIT 18.2) or an *in vitro* kinase reaction in which purified kinases (possibly immunoprecipitated) are used to phosphorylate crude or fractionated (sometimes coimmunoprecipitated) cell lysates using  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  (UNIT 18.7). The mixture is then separated by gel electrophoresis and the migration of the labeled protein detected by autoradiography. The type of amino acid residues modified can be identified by mild base hydrolysis of the excised proteins, and separation of the amino acids by thin-layer chromatography (TLC) or electrophoresis (UNIT 18.3). To determine the site of modification, the protein must be fragmented and the resulting peptides separated, usually by two-dimensional TLC with autoradiographic detection, or sometimes by RP-HPLC with Cerenkov counting of collected fractions. Again, the type of residue phosphorylated can be determined by mild base hydrolysis followed by one- or two-dimensional TLC (see UNIT 18.3). Alternatively, the site can be revealed by Edman sequencing of the fractionated peptide, in the rare cases where sufficient sample is available, or by comigration of synthetic phosphorylated peptides designed from potential sites of phosphorylation within the protein of interest. For both of these approaches to work the amino acid sequence of the protein has to be known. The ability to raise antisera to phosphotyrosine has provided a very simple nonradioactive means of identifying proteins carrying this modification (see UNITS 18.5 & 18.6). Antibodies are available from several commercial sources, providing a rapid and sensitive means of monitoring tyrosine phosphorylation on SDS-PAGE (including two-dimensional gel electrophoresis) separated proteins. Antisera directed against phosphoserine and phosphothreonine detect some but not all proteins modified in this manner.

## MS Approaches for Identifying Regions and Sites of Phosphorylation

A number of mass spectrometry-based approaches have been developed to address the issue of protein phosphorylation. They fall into two main categories: searching for mass differences consistent with phosphorylation (i.e.,

+80 u or multiples thereof) and then treating with phosphatases to confirm the assignment, and searching for phosphorylated peptides through the use of high-orifice scanning in ESI-MS to find phosphate-specific signature ions (see UNIT 10.21). Although it is always preferable to determine the intact mass of the protein as the first step in analysis, this is often not possible; therefore, the protein is enzymatically digested and the masses of the resulting peptides are determined.

If the entire sequence of the protein under investigation is known, then putative phosphorylated peptides can be assigned based upon which peptides do not match the expected masses. Of course, this only localizes the site of modification, but doesn't confirm that the expected residue is modified with phosphate; that requires sequencing of the peptide either chemically or by MS/MS. Confirmation of the observed mass difference can be made by treating the digest with a phosphatase and measuring the peptide masses again. This can be conducted with a separate aliquot in solution, or as Craig et al. (1994) and Liao et al. (1994) have demonstrated for MALDI-MS, directly on the probe, making the analysis rapid and with minimal sample loss. If the putative assignments were correct, those previously phosphorylated peptides will now display masses 80 u, or multiples thereof, less than the first analysis. This seems relatively straightforward, but the approach may not always result in complete assignment of all phosphorylated sites. Independent of the mass spectrometer used (MALDI-MS or ESI-MS), not all of the peptides generated from the digest may be able to be mass analyzed, e.g., some peptides may only be ionized in either positive- or negative-ion mode but not both, or some peptides may become insoluble following digestion. Mass spectrometry of peptides is usually conducted in positive-ion mode (see UNIT 10.21), but because phosphorylation is a negatively charged modification, it sometimes changes the properties of the peptide such that it will only be ionized in negative-ion mode. For MALDI-MS, conducting experiments in both modes sequentially is not a problem because the sample is immobilized, and many analyses can be performed on the same sample. In ESI-MS, the analysis is usually performed in one mode (positive or negative) or the other.

To overcome the problem of incomplete coverage of the protein due to insolubility or other problems, a parallel digest with an enzyme of differing specificity is often used. In

an elegant study of a PAK/STE20 homolog from *Acanthamoeba castellanii*, Szczepanowska et al. (1997) used MALDI-MS on both a TOF and an ion-trap instrument to identify the site of in vivo modification by analysis of unfractionated tryptic and Glu-C digests. In this study, all of the possible sites of phosphorylation were observed as tryptic or Glu-C-derived peptides, and the one site of phosphorylation was identified in both the baculovirus-expressed truncated form of the protein as well as the endogenous full-length form. Both phosphatase treatment and MS/MS fragmentation were employed for confirmation that the peptide was phosphorylated and to determine the site of modification.

In the second approach, advantage was taken of the observation that under ionization conditions that leave most peptide bonds intact, phosphate ester bonds in phosphopeptides can be dissociated at the source (or in a collision cell) of an ESI-MS. When the analysis was conducted in negative-ion mode (the potential on the spraying needle is negative) with the orifice voltage at a high potential (e.g., -350 V), phosphopeptides containing phosphoserine or phosphothreonine yielded signature low-mass fragment ions at 97, 79, and 63 u, and phosphotyrosine-containing phosphopeptide low-mass ions at 79 and 63 u. In the original methods of Huddleston et al. (1993, 1994) and Ding et al. (1994), peptide digests were separated by RP-HPLC. On-line ESI-MS was employed to determine both the mass of the peptide as well as its phosphorylation status by switching the voltage on the orifice between a high potential when scanning (measuring masses) in the low-mass region, to a normal potential when scanning over the remainder of the mass range, in a cyclical manner. Of course, fractions can be collected during the run for subsequent analysis. This is a rapid method to identify which peptides in a given mixture are phosphorylated, as well as what their intact masses are (unless the mixture is too complex and many peptides coelute on the RP-HPLC). However, as with the MALDI-MS approach, this only localizes the modified residues and does not confirm the site of modification until the fractionated peptides are sequenced either chemically or by MS/MS. Another feature of the approach is that even when the RP-HPLC is conducted on a 320- $\mu$ m-i.d. capillary column, the limit of detection is suggested to be 50 pmol (Huddleston et al., 1993), thereby limiting the RP-HPLC/ESI-MS approach to in vitro phosphorylation site analysis.

The ability to spray sample into the ESI-MS at very low flow rates (so called nanospray, due to low nanoliter per minute flow rates; Wilm and Mann, 1994), has dramatically increased the sensitivity of MS/MS sequencing. This method can also be used to advantage for post-translational modification analysis of *in vivo*-generated samples due to the high sensitivity. In addition to the method where high-orifice scanning generates signature low-mass ions for phosphorylated residues, another MS/MS-based approach can also be used in conjunction with a triple quadrupole instrument (see UNIT 10.21). This instrument can be run in a number of modes, two of which are described here. In normal MS/MS mode to generate fragment ions from a specific precursor ion (peptide), the first set of quadrupoles (Q1) is set to allow transmission of only the peptide ion of interest into Q2 which in turn is set to transmit all ions, not just a specific  $m/z$  into which a curtain of gas (e.g., argon) has been injected. The peptide ions undergo collision-induced decomposition (CID) into sequence-specific fragment ions, whose masses can be determined by scanning Q3. Thus, the fragment ions from a specific precursor ion can be generated and measured. In a mode referred to as precursor (or parent)-ion scanning, the third set of quadrupoles (Q3) are set to transmit only a specific  $m/z$ ; in this example for identification of phosphorylated peptides in a mixture, this is set to  $m/z = 79$ . The collision gas is left on in Q2, and ions are scanned over the mass range in Q1. Therefore, a signal will only be recorded when a fragment ion of 79 u is generated from a parent ion of the mass being scanned at that point in time in Q1. A spectrum is produced of any peptide that following CID produces a fragment ion of 79 u, i.e., a phosphopeptide. The advantage of this approach of precursor-ion scanning is that the phosphopeptides can be selected from a mixture for fragmentation analysis in the normal mode (i.e., select in Q1, fragment in Q2, mass analyze in Q3).

This approach has been employed successfully by Betts et al. (1997) in their identification of sites of *in vivo* phosphorylation on neurofilament proteins (NF-M and NF-L) isolated from rat brain. They separated the neurofilament-enriched fraction from rat brain by SDS-PAGE, digested the protein in the gel, desalted it, and performed nanoESI-MS/MS on the resulting mixtures. They also analyzed peptide mixtures that had been enriched in phosphopeptides by binding the in-gel digest eluant to an immobilized metal-affinity column

(IMAC; UNIT 10.11B) equilibrated with  $\text{FeCl}_3$  followed by elution with Tris·Cl (Betts et al., 1997). They analyzed not only the phosphopeptides but all of the peptides possible to determine whether the phosphorylation was heterogeneous at the identified sites. By using three enzymes, trypsin, Glu-C, and Asp-N, they achieved 81% sequence coverage for NF-L and 64% sequence coverage for NF-M. Interestingly, no sites of phosphorylation were found on NF-L, and the four sites identified on NF-M were shown to be heterogeneous.

### Automated Approaches for Identifying Phosphorylation Sites

The nanoESI-MS/MS approach described above provides high-sensitivity analytical capability for both protein identification and post-translational modification determination. The drawbacks are that setup of the device is not rapid (although a number of simpler devices are now becoming available), and the analysis is manual, i.e., an operator manually sets the instrument in all different modes and selects peptides for fragmentation. Other groups have been investigating automating posttranslational modification analysis by constructing integrated on-line systems. These consist of an RP-HPLC-ESI-MS/MS (LC-MS/MS) system for automated MS/MS of separated peptides, with an additional column on-line in front of the RP-HPLC system which provides the specificity to the system. Such columns include an IMAC column (for general capture of phosphorylated peptides), immunoaffinity and SH2-domain affinity column (phosphotyrosine specific), and an enzyme reactor (a tyrosine phosphatase). A general schema for such devices is shown in Figure 10.22.5 and a brief description of these approaches follows.

Watts et al. (1994) used an on-line IMAC column in front of a LC-MS to capture phosphopeptides in a protocol that resulted in determination of the sites of *in vivo* tyrosine phosphorylation of the lymphocyte-specific protein tyrosine kinase ZAP-70. In this approach, standard two-dimensional TLC phosphopeptide maps were produced for both *in vivo* phosphorylated ZAP-70 and recombinant ZAP-70 phosphorylated with recombinant p56lck *in vitro*. From the *in vitro* generated two-dimensional TLC plate, spots were excised, the slurry extracted and recovered peptides subjected to IMAC-LC-MS to determine the mass of the phosphopeptides and identify the sites of phosphorylation. Comparison (including comigration of excised spots) of the *in vivo*- and *in*

vitro-generated two-dimensional TLC patterns allowed identification of the sites of autophosphorylation and T cell receptor-stimulated phosphorylation. The sensitivity of the system was demonstrated to be ~250 fmol (Watts et al., 1994). This approach provides a high-sensitivity system that complements the extensively utilized two-dimensional TLC mapping procedure (Watts et al., 1996).

Specific capture of tyrosine-phosphorylated peptides has been achieved using an antiphosphotyrosine column in-line with an LC-MS (Kassel et al., 1995). The specificity of the system was demonstrated, but the sensitivity of the approach was not determined. Kassel et al. (1995) also produced a more specific affinity column by immobilizing a recombinant SH2 domain to the first column, thus allowing capture of only tyrosine phosphopeptides containing the specific sequence motif recognized by this domain. Amankwa et al. (1995) developed an "enzyme reactor" system employing a broad-specificity tyrosine phosphatase on-line prior to either capillary zone electrophoresis (CZE)-MS or LC-MS. Comparison of peptide maps with and without passage through the enzyme reactor allowed determination of sites of tyrosine phosphorylation at low levels (2.6 pmol).

### Role of MS in Posttranslational Modification Analysis

More than 400 posttranslational modifications have been described to date (Krishna and Wold, 1993), and mass spectrometry has been used extensively in the characterization of many of them (for a list of posttranslational modifications and their masses see APPENDIX 1B, Table A.1C.4, or for an extensive on-line list see Dr. K. Mitchell-Hill's Delta Mass site at <http://www.medstv.unimelb.edu.au/WWWD/OCS/SIVMRDocs/MassSpec/deltamassV2.html>). However, mass spectrometry has yet to be employed in a high-throughput mode for a variety of modifications, and in many cases it is not the primary method of choice in characterization of posttranslational modifications. With the increased sensitivity, availability, and ease of use of these instruments, mass spectrometry will be used more and more in routine analyses to detect and characterize posttranslational modifications. As stated previously, characterization of the posttranslational modifications present on a protein requires an initial measurement of the intact mass of the protein. For electrophoretically separated proteins, this has proven to be a difficult task at low levels

when the protein is eluted from the gel slice for subsequent ESI-MS analysis. Although a few protocols recently have been demonstrated for mass analysis of low-picomole quantities of pure protein eluted from gels (e.g., Schuhmacher et al., 1996; Cohen and Chait, 1997), the methods described previously for direct mass measurement of gel-separated proteins either off membranes or out of gels may simplify this first step in the analysis. Enzymatic digestion and analysis of the resulting fragments allows the region of modification to be determined, and generation of fragment-ion spectra from these peptides in most cases will assign the site of modification. Thus, the ability to characterize small quantities of gel-separated proteins is close to becoming reality.

An extremely promising advance in post-translational modification analysis is the demonstration by Bean et al. (1995) of the lability of a number of posttranslational modifications to stepped high-orifice scanning in LC-MS analyses as described for phosphorylation analysis. This approach results in the generation of signature ions for specific modifications to indicate their presence on peptides eluting at that retention time, and in combination with MS analysis at normal orifice voltage, the intact mass of the modified peptides. Modifications amenable to stepped high-orifice scanning include phosphorylation, palmitoylation, sulfation, and glycosylation (Bean et al., 1995). In the case of glycosylation the presence of individual classes of carbohydrates present on individual peptides—hexose, hexosamine, and neuraminic acid—are revealed. Fragmentation of the peptide in an automated mode may reveal the site of modification. However, in some cases (e.g., glycosylation), the modifying group has to be removed for sequence-specific fragment-ion data to be obtained.

Identification of the site of modification is critical to complete analysis, but if the modification is not stable to the mass spectrometry ionization or fragmentation process, then the modifying group needs to be removed and in doing so, "tag" the site from which it was removed. In this case the peptide is then fragmented and the site of former modification identified. Gonzalez et al. (1992) used this approach to identify sites of N-glycosylation by using the enzyme peptide-N-4(N-acetyl-β-glucosaminyl)asparagine amidase F (also known as PNGase F) in the presence of 50% H<sub>2</sub><sup>18</sup>O. PNGase F completely removes the carbohydrate side chain from the asparagine residue and in so doing results in conversion of this



residue to aspartic acid, which is 1 u heavier than asparagine following incorporation of H<sub>2</sub>O from the solvent (see UNIT 17.14A). By conducting the reaction in 50% (v/v) H<sub>2</sub><sup>18</sup>O, 50% of the oxygen atoms incorporated during the removal of the carbohydrate side chain and conversion of asparagine to aspartic acid will have a mass 2 u greater than normal aspartic acid (see Fig. 10.22.6). Therefore, a pair of peptides differing in mass by 2 u will be produced following this reaction. MS/MS analysis of this peptide pair will result in doublets 2 u apart for all fragment ions containing the site of modification thereby allowing identification of the site of former modification (Gonzalez et al., 1992).

MS/MS analysis of peptides containing O-linked sugar or phosphate on serine and threonine residues does not always allow unambiguous determination of the site of modification due to the lability of the O-linked modifying group; often significant neutral loss occurs during MS/MS, complicating the analysis. However, removal of either of these modifying groups by  $\beta$ -elimination results in dehydration of the serine and threonine residues, leaving unique residue masses for each, 18 u less than normal (see Fig. 10.22.6; see UNIT 17.15B for  $\beta$ -elimination protocols applied to glycosylated peptides). This has been applied to determination of sites of O-GlcNAc modification of intracellular proteins and to characterize sites of phosphorylation in the highly phosphorylated profilaggrin (Resing et al., 1995; Greis et al., 1996). In both cases, the altered residue mass (–18 u for each site of modification) was used to localize the peptides upon which the modification occurred, and the site was identified by MS/MS. Greis et al. (1996) demonstrated the ability to determine the intact mass of glycopeptides by LC-MS analysis of the native sample and, following  $\beta$ -elimination, the site of glycosylation on the peptides. Resing et al. (1995) noted that MS/MS fragmentation was more pronounced at the peptide bonds either side of the dehydrated residue thus facilitating analysis of the fragment-ion spectrum.

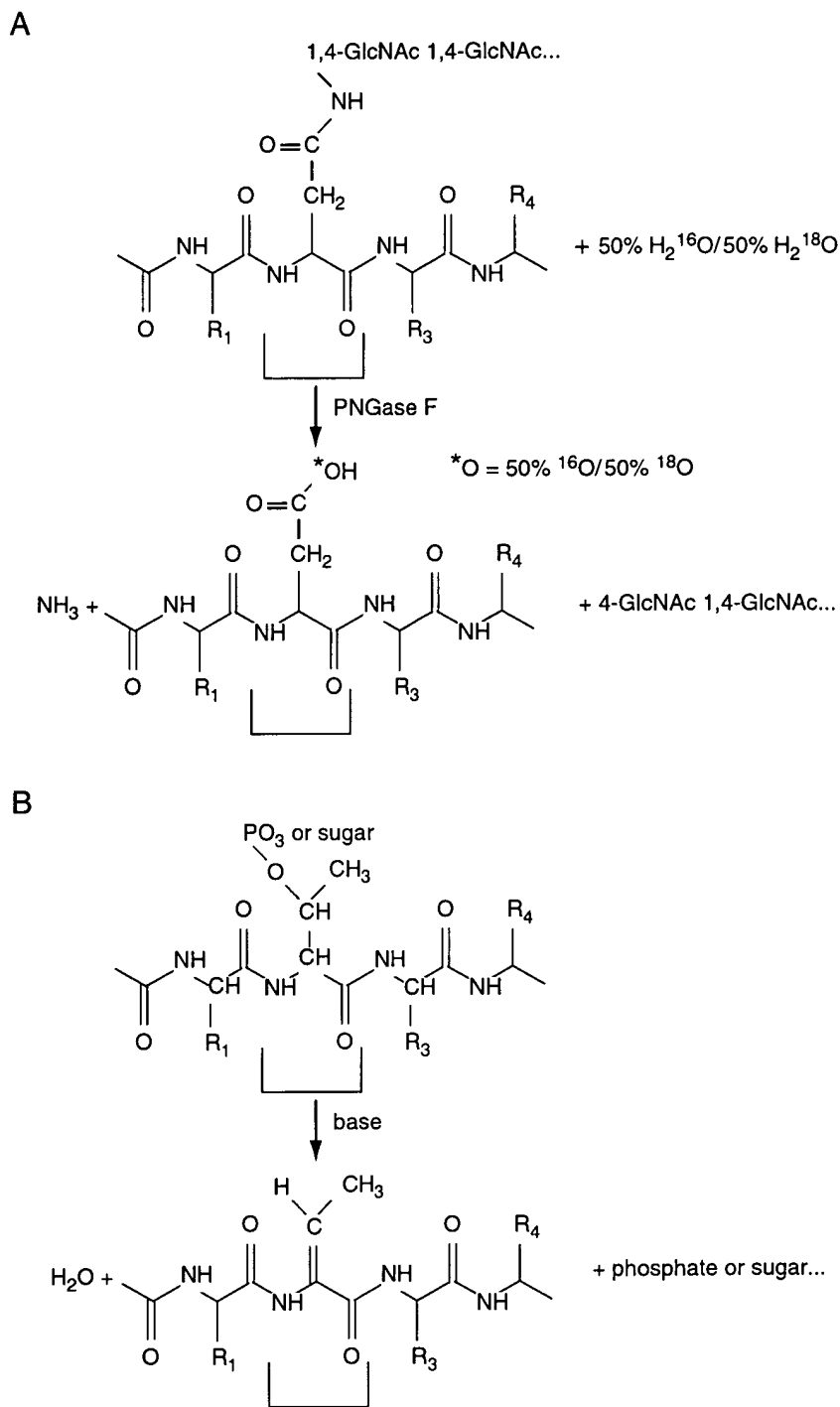
Tagging sites of modification by changing the mass of the amino acid carrying the modifying group provides a powerful tool in determining the site of modification. Conducting MS and MS/MS analysis of the sample prior to removal of the group also allows some characterization of the modifying group. Interestingly it has recently been demonstrated that mixtures of N-linked oligosaccharide structures can be determined from gel-separated proteins at the

low microgram level by in-gel PNGase F digestion followed by MALDI-MS analysis, after which the remaining protein can be digested for subsequent peptide-mass mapping experiments (Kuster et al., 1997).

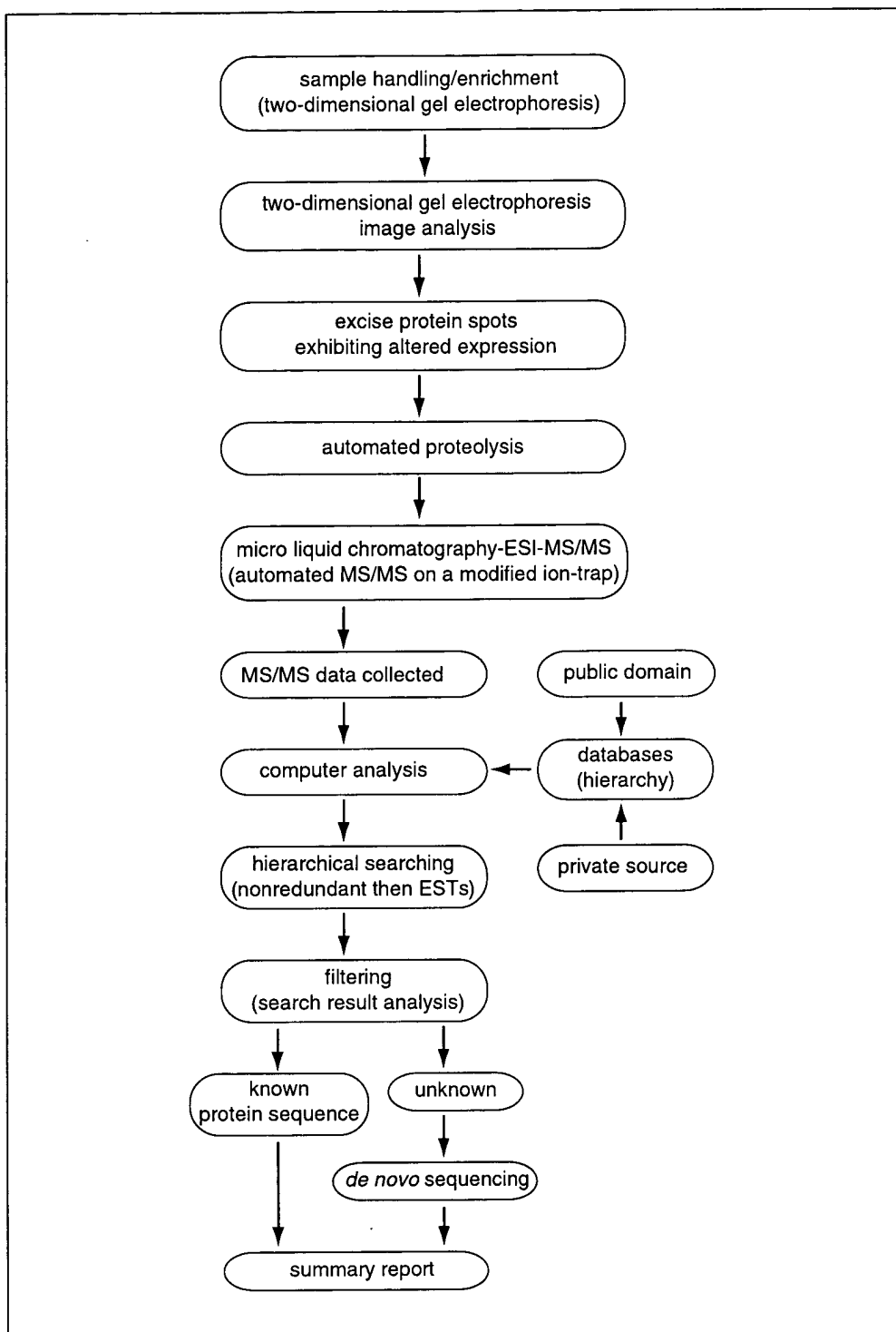
## HIGH-THROUGHPUT PROTEIN IDENTIFICATION

The highest-resolution protein separation method is still two-dimensional gel electrophoresis. Two-dimensional gel electrophoresis provides the ability to display literally thousands of proteins in one experiment, revealing their relative levels of expression and, depending on how the sample was prepared, their subcellular localization and predetermined posttranslational modifications (i.e., by radiolabeling with specific metabolic precursors). Since the method of two-dimensional gel electrophoresis was first introduced in 1975 (Klose, 1975; O'Farrell, 1975; Scheele, 1975; also see UNIT 10.3), a frustrating aspect has been the inability to identify individual proteins and extensively characterize their modifications. This unit and others in this volume (and *Current Protocols in Protein Science*) describe the increasing ability to identify minute quantities of gel-separated protein, but large-scale identification of many proteins in a two-dimensional gel electrophoresis pattern has only recently begun to be addressed successfully.

One semiautomated approach uses existing methods for parallel enzymatic digestion of excised spots and automated loading of a fraction of the digest onto a MALDI-MS plate for subsequent unattended data generation and capture followed by peptide-mass searching (Shevchenko et al., 1996). This approach was used in a yeast proteome project where proteins isolated by two-dimensional gel electrophoresis were identified using a two-part strategy: peptide-mass searching employing an automated 32-port parallel enzyme digestion robot (Houthaeve et al., 1995), followed by automated MALDI-MS with delayed-extraction of a small aliquot of the peptide digest and subsequent automated peptide-mass searching. If this didn't conclusively identify the protein, nano-ESI-MS/MS to generate a sequence tag was undertaken using half of the remaining sample after it was reduced in volume and cleaned up using a microdesalting step. Peptide-sequence tags generated from a number of peptides in the mixture were then used in searches to identify the protein. If this was not successful, then the remainder of the digest could be derivatized by methyl esterification



**Figure 10.22.6** "Tagging" sites of posttranslational modification for mass spectrometric analysis. **(A)** Tagging sites of N-linked glycosylation. PNGase F is used to remove N-linked carbohydrate side chains in the presence of 50% H<sub>2</sub><sup>18</sup>O (50% normal water) changing the asparagine residue (residue mass 114 u) to an aspartic acid residue (which exists as a doublet of residue masses 115 u and 117 u). **(B)** Tagging sites of O-linked phosphorylation or glycosylation.  $\beta$ -elimination (base hydrolysis) will remove O-linked phosphate or sugar from threonine (residue mass 103 u, shown) or serine (residue mass 87 u) residues and in doing so dehydrate the side chain resulting in a 18 u decrease in mass to yield dehydroamino-2-butyric acid (residue mass 83 u) and dehydroalanine (residue mass 69 u) respectively. The amino acid residues of interest are bracketed.



**Figure 10.22.7** A scheme for automated identification of proteins exhibiting altered expression when assayed by two-dimensional gel electrophoresis (adapted from figures supplied by Dr. R. B. Parekh, Oxford GlycoSciences, Abingdon, UK). The samples to be analyzed are fractionated as appropriate prior to two-dimensional gel electrophoresis separation using an automated system. The two-dimensional gel electrophoresis-resolved proteins are imaged and differences between samples highlighted by a computer system, which then excises protein spots from the gel and digests them—all under robotic control. The digest is then separated by micro-LC-ESI-MS/MS in an automated mode, and the MS/MS spectra are transferred to a database from which automated searches are conducted against protein and translated nucleotide (including ESTs) databases. If the sequence resides in a database, the match is determined; if not, the MS/MS pattern is used to interpret the sequence *de novo*.

and subjected to an additional nano-ESI-MS/MS analysis to interpret the sequence *de novo* from the fragment-ion spectra (Shevchenko et al., 1996). This approach was able to identify 150 yeast proteins separated by two-dimensional gel electrophoresis in a high-throughput mode.

A fully automated system has been developed by Oxford GlycoSciences (Dr. R. B. Parekh, pers. comm.), where two-dimensional gels are run under computer control followed by automated image analysis of fluorescently labeled proteins. This allows the user to select spots of interest on a computer screen for automated excision, digestion, LC-MS/MS, and SEQUEST software analysis (see Fig. 10.22.7). For large-scale two-dimensional gel electrophoresis projects this type of approach can be advantageous, and it fulfills the requirements of a proteomics approach to two-dimensional gel electrophoresis-separated proteins. However, these approaches are most likely to be adapted only in core facilities or even more specialized laboratories.

When the mixtures are less complex, a chromatography-based approach has been demonstrated to provide rapid automated protein identification without employing any steps of electrophoresis. All of the approaches described so far analyze peptides derived from one or a few proteins and therefore, a number of fragment-ion spectra are obtained from each protein, and these allow redundant identifications. Other automated approaches rely upon only one peptide fragment-ion spectrum for identification as the protein mixture is digested prior to peptide separation. This provides a simpler approach for automation, but at least one peptide from each protein in the mixture must be analyzed for a completely successful analysis to be performed.

Enzymatic digestion of relatively complex protein mixtures followed by data-dependent LC-MS/MS (i.e., the mass spectrometer selects the peptides on which to perform MS/MS on-the-fly) and automated uninterpreted fragment-ion searching of sequence databases using the SEQUEST program, has been demonstrated to be capable of identifying many components within a mixture (McCormack et al., 1997). The key to the success of such an approach is the ability to isolate mixtures of limited complexity that are of biological interest. Yates and colleagues (McCormack et al., 1997) demonstrated identification of components of a co-immunoprecipitation (see UNIT 10.16), a micro-

tubule complex, as well as a set of protein standards, to demonstrate the ability to detect a component at lower concentration in a mixture where the other components were at a much higher level. This analysis revealed that fragment-ion spectra from only a few peptides were usually obtained, but of course, in using this approach only one MS/MS spectrum needs to be obtained per protein if the spectral quality is good enough. The main advantage of this approach is the speed with which the analysis can take place compared even with automated two-dimensional gel electrophoresis approaches and the relative simplicity of the setup. The described system uses capillary LC which allows low amounts of proteins to be analyzed. The disadvantages of the approach include: (1) difficulty in comparing relative protein levels, aside from presence or absence; (2) generation of MS/MS data from contaminants when they could have been excluded from analysis if a comparative (e.g., electrophoretic) analysis had been performed; and (3) the limited number of components that can be analyzed in one experiment. However, given these caveats there are still many applications that could benefit from an analytical approach capable of rapidly identifying a mixture of protein components through analysis of their derived peptides. For example, in addition to the examples presented above, identification of the major components present during a specific purification step may allow the purification to be directed to remove unwanted components. An increased number of peptide components could be analyzed if a two-dimensional chromatographic system were employed.

Opiteck et al. (1997b) described a two-dimensional chromatography system where peptides were separated by size-exclusion chromatography (SEC; UNIT 10.14) and loaded alternately onto one of two HPLC columns for sequential, rapid RP-HPLC separation followed by ESI-MS analysis. Four-minute SEC fractions were loaded onto the RP-HPLC column, and while this was being separated over 3 min (with 1 min reequilibration), the other HPLC column was being loaded. The two HPLC columns were run alternately over 160 min, each running 20 gradients in that time (40 consecutive gradients were run by the one set of HPLC pumps). This approach provided a much greater resolving power than either method alone and in an automated format (Opiteck et al. 1997b). Of course, such a system could be used in conjunction with an ESI-MS

system capable of data-dependent fragmentation, yielding fragment-ion spectra for each peptide component as well.

In an extension of the LC-MS/MS method, additional on-line steps have been added at the beginning to automate the entire analysis by allowing (1) immunoaffinity chromatography purification, (2) desalting and buffer exchange on a mixed-bed strong ion-exchange absorbent, (3) digestion on an immobilized trypsin column, (4) capture of the tryptic peptides on a short perfusion capillary reversed-phase column, and (5) separation on an analytical reversed-phase column with the column effluent being analyzed by ESI-MS/MS (Hsieh et al., 1996). The entire computer-controlled setup requires an autosampler, five columns, and three 10-port switching valves to allow all of the steps to be performed on-line without any manual transfers of material (Hsieh et al., 1996). The method was described only for analysis of protein purified by the system, but it may be able to be extended to the analysis of many components of a complex protein mixture.

## CONCLUSION

This unit has provided the reader with a glimpse into some new technologies that have been developed for protein analysis. Not all aspects of protein analysis have been covered, with an emphasis being placed on protein identification and characterization. However, it is hoped that the reader will have a better understanding of how mass spectrometry techniques can be used to answer questions of biological importance both rapidly and accurately. Proteome analysis in its broadest definition should provide evidence of the steady-state level of expression for all individual gene products in a cell and their modifications at a defined point in the life of the cell. In its simplest form, it can be used just to confirm open reading frames in DNA sequences. In addition, unless the cell population is truly synchronous, such an analysis needs to be undertaken on a very small number of cells. Unless radiolabelling of newly synthesized proteins is performed, only the steady-state levels of proteins can be determined. In addition to expression levels, the subcellular localization of each protein needs to be identified, and this is an extremely complex process requiring many parallel experiments. Such an analysis is still far from being realized without using a large number of experimental protocols. However, with the tools currently in hand, the prospect of attaining such

a goal in a reasonable time frame looks a little less daunting.

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# Identification of Low Density Lipoprotein Receptor-related Protein-2/megalin as an Endocytic Receptor for Seminal Vesicle Secretory Protein II\*

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The low density lipoprotein receptor-related protein-2/megalin (LRP-2) is an endocytic receptor that is expressed on the apical surfaces of epithelial cells lining specific regions of the male and female reproductive tracts. In the present study, immunohistochemical staining revealed that LRP-2 is also expressed by epithelial cells lining the ductal region and the ampulla of the rat seminal vesicle. To identify LRP-2 ligands in the seminal vesicle, we probed seminal vesicle fluid with <sup>125</sup>I-labeled LRP-2 in a gel-blot overlay assay. A 100-kDa protein (under non-reducing conditions) was found to bind the radiolabeled receptor. The protein was isolated and subjected to protease digestion, and the proteolytic fragments were subjected to mass spectroscopic sequence analysis. As a result, the 100-kDa protein was identified as the seminal vesicle secretory protein II (SVS-II), a major constituent of the seminal coagulum. Using purified preparations of SVS-II and LRP-2, solid-phase binding assays were used to show that the SVS-II bound to the receptor with high affinity ( $K_d = 5.6$  nM). The binding of SVS-II to LRP-2 was inhibited using a known antagonist of LRP-2 function, the 39-kDa receptor-associated protein RAP. Using a series of recombinant subfragments of SVS-II, the LRP-2 binding site was mapped to a stretch of repeated 13-residue modules located in the central portion of the SVS-II polypeptide. To evaluate the ability of LRP-2 to mediate <sup>125</sup>I-SVS-II endocytosis and lysosomal degradation, ligand clearance assays were performed using differentiated mouse F9 cells, which express high levels of LRP-2. Radiolabeled SVS-II was internalized and degraded by the cells, and both processes were inhibited by antibodies to LRP-2 or by RAP. The results indicate that LRP-2 binds SVS-II and can mediate its endocytosis leading to lysosomal degradation.

The low density lipoprotein receptor-related protein-2/megalin (LRP-2)<sup>1,2</sup> has been shown to be widely expressed by cells

lining the reproductive tracts of male and female rodents (1–3). For example, it is expressed by epithelial cells of the efferent ducts, epididymis, and vas deferens of the male and by the germinal layer and epithelium of endometrium and oviduct of the female (1–4). The function of LRP-2 in the reproductive tracts is not yet known, but an endocytic function can be inferred from its apical surface localization and presence within endocytic compartments including coated pits, endocytic vesicles, and early endosomes of efferent duct cells and principal cells of the epididymis (2).

During transit through the epididymis, spermatozoa undergo a maturational process that includes morphological and physiological changes (e.g. acquisition of sperm motility and alterations in the properties of the plasma membrane). A widely held view is that the secretory and absorptive activity of the epithelial cells lining the efferent ducts, epididymis, and accessory glands (e.g. seminal vesicles) profoundly influences sperm maturation (5–7). Given the prominent expression of LRP-2 by efferent duct and epididymal epithelial cells, it has been hypothesized that LRP-2-mediated catabolism of proteins present in the epididymal fluid and/or associated with spermatozoa surfaces may be a contributing factor to spermatozoa maturation (2). Indeed, many LRP-2 ligands are found in the epididymis, seminal vesicle, and seminal plasma such as apolipoprotein J/clusterin (2), apolipoprotein B (8), apolipoprotein E (9), tissue-type plasminogen activator, urokinase-type plasminogen activator, and plasminogen activator inhibitor-I (10) and have been speculated to be involved in sperm maturation and fertilization.

We report here that LRP-2 is also expressed by epithelial cells of the seminal vesicles. In the rat, the seminal vesicles are two coiled tubular glands connected by a short duct to an excretory tubule that is anatomically equivalent to the ampulla of humans and other primates and connected to the ejaculatory duct (11). The seminal vesicles produce proteins that become covalently cross-linked by a transglutaminase and form the fibrin-like seminal coagulum. In rodents, the seminal coagulum forms the copulatory plug, whereas in humans, seminal coagulum is degraded within the female reproductive tract by proteases of prostatic origin, such as prostate-specific antigen (PSA), a member of the kallikrein family of serine proteinases (12, 13). The major components of the seminal coagulum belong to a multigene family (14, 15) that includes proteins such as rat seminal vesicle secretory proteins II, IV, and V (SVS-II, IV, and V, respectively) (16, 17), mouse seminoclotin (14), and human seminogelins I and II (18). In the following study, we present evidence indicating that a major rodent seminal vesicle secretory protein and transglutaminase substrate, SVS-II, is a novel LRP-2 ligand.

\* LRP-2 is synonymous with glycoprotein 330 (gp330).

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¶ The abbreviations used are: LRP-2, low density lipoprotein receptor related protein-2/megalin; RAP, receptor-associated protein; SVS-II, seminal vesicle secretory protein II; TBS, Tris-buffered saline; Bt<sub>2</sub>cAMP, dibutyryl cyclic AMP; DAB, 3,3'-diaminobenzidine tetrahydrochloride; GST, glutathione S-transferase; DMEM, Dulbecco's modified Eagle's medium; RA, retinoic acid.

TABLE I  
Oligonucleotide primers used in the generation of SVS-II cDNAs

Primer no.	Oligonucleotide sequence <sup>a</sup>	Positions <sup>b</sup>
1	<u>CCCGGGAGGTGGGACAAAAGGTCAC</u>	2376–2393
2	<u>GAATTCCTTCTGGGATTCAATTGGGATAC</u>	2619–2596
3	<u>CCCGGGATCCCAAATAAAATCCTTTAGAC</u>	2627–2349
4	<u>GAATTCGAGGGATTTTGTGTGCCAAAGGAC</u>	3236–2313
5	<u>CCCGGGAAAAGGGTTTCTCAACAACTC</u>	3237–3256
6	<u>GAATTCCTTATCCCTTAAGTTGAGCCTG</u>	3548–3528

<sup>a</sup> Restriction site sequences (*Sma*I and *Eco*RI) and residues added to maintain reading frame are underlined.

<sup>b</sup> The numbers indicated correspond to nucleotide residues within the rat SVS-II sequence (GenBank™ accession number J05443).

#### EXPERIMENTAL PROCEDURES

**Proteins**—LRP-2 was purified from porcine kidney using RAP-Sepharose affinity chromatography as described previously (19). Human 39-kDa receptor-associated protein (RAP) was expressed as a glutathione S-transferase fusion protein in bacteria and was purified according to Williams *et al.* (20). SVS-II was purified from rat seminal vesicle as described by Wagner and Kistler (16). Subfragments of rat SVS-II corresponding to the N-terminal domain (from residues 25 to 107), 13-residue-repeat domain central domain (from residues 108 to 311), and C-terminal domain (from residues 312 to 414) were expressed in bacteria as fusion proteins with GST using the pGEX2T vector (Amersham Pharmacia Biotech) using primers described in Table I. Fusion proteins were purified by glutathionine-Sepharose (Amersham Pharmacia Biotech) chromatography as described by the manufacturer. Fibulin-1 was purified from human placenta as described in Argraves *et al.* (21). LRP-2 and SVS-II were labeled with [<sup>125</sup>I]iodine (Amersham Pharmacia Biotech) using Iodogen (Pierce Chemical Co., Rockford, IL) to specific activities of 7–8 and 2–10  $\mu$ Ci/ $\mu$ g, respectively (~0.222 MBq/ $\mu$ g and ~0.279 MBq/ $\mu$ g, respectively).

**Antibodies**—LRP-2-Sepharose affinity chromatography was used to isolate anti-LRP-2 IgG from a polyclonal serum (rb6286) derived from a rabbit immunized with porcine kidney LRP-2 prepared as described previously (22). The bound IgGs were eluted using both high and low pH buffers and dialyzed against TBS (150 mM NaCl, 50 mM Tris, pH 7.4). After dialysis, the IgG preparation was applied to a column of RAP-Sepharose (1.0 mg of protein/ml of resin) and the unbound IgG selected on protein G-Sepharose. Control IgG was purified from normal rabbit serum using protein G-Sepharose.

Mouse monoclonal antibody 3C3 to human LRP-2 was prepared by fusing NS1 myeloma cells with spleen cells from Balb/c mice immunized with human LRP-2 purified from urine (23). Hybridomas were selected based on production of immunoglobulin G antibodies reactive with both human and rat LRP-2 by both enzyme-linked immunosorbent assay (ELISA) and immunoblotting.

**Isolation of Rat Seminal Vesicle Fluid**—Sexually inactive, male Sprague-Dawley rats (age 6–8 months) were killed by CO<sub>2</sub> inhalation, and their seminal vesicles were removed. The fluid contents of the seminal vesicles were released immediately into an ice-cold solution of spermine and spermidine (Sigma) (each at 5 mM in water). The fluid-containing solution was centrifuged for 10 min at 10,000  $\times$  g, and the supernatant was recovered. Protein concentration in the clarified fluid was estimated using a commercial protein assay system (Bio-Rad).

**Cells**—Mouse embryonic F9 teratocarcinoma cells (ATCC CCL 185) were obtained from American Type Culture Collection and grown on 176-cm<sup>2</sup> plates (Corning, Corning, NY) coated with 0.1% gelatin (Sigma) in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Inc.) with 10% bovine calf serum (HyClone Laboratories, Logan, VT), penicillin, and streptomycin (Mediatech, Herndon, VA). To augment LRP-2 expression, the cells were treated with 0.1  $\mu$ M RA (Calbiochem, San Diego, CA) and 0.2  $\mu$ M Bt<sub>2</sub>cAMP (Sigma) for 6–7 days without a change of medium (24).

**Immunohistochemistry**—Isolated rat seminal vesicles were dissected into four parts along their proximal to distal axis. Each segment was placed in Bouin's fixative (EM, Diagnostic Systems, Gibbstown, NJ) for 4 h at room temperature and then placed in 70% ethanol for 18 h at 4 °C. The fixed tissue specimens were dehydrated, embedded in paraffin, and sectioned at 5  $\mu$ M thickness. Immunohistochemical staining

was carried out using a Sequenza coverplate system (Shandon Lipshaw, Pittsburgh, PA) with polyclonal LRP-2 IgG (rb6286) and normal rabbit IgG (each at 2  $\mu$ g/ml). An Elite Vectastain ABC kit (Vector Laboratories, Burlingame, CA) and the chromogenic substrate DAB (Sigma) were used for antibody detection. Sections were counter-stained with hematoxylin (Shandon Lipshaw).

**Gel-blot Overlay Assay**—Aliquots of rat seminal vesicle fluid, rat SVS-II, GST-SVS-II fusion proteins, GST, RAP, or fibulin-1 were electrophoresed with fibulin-1 and RAP on 4–12% polyacrylamide gels (Novex, San Diego, CA) in the presence of SDS and under non-reducing conditions. The separated proteins were electrophoretically transferred onto nitrocellulose membranes (Schleicher and Schuell), and unoccupied sites were blocked with 3% nonfat milk in TBS (pH 8.0) (blocking buffer). The membranes were incubated with <sup>125</sup>I-LRP-2 (1 nM) in blocking buffer containing 5 mM CaCl<sub>2</sub> and 0.05% Tween 20. To block the function of LRP-2, radiolabeled LRP-2 solution was preincubated with RAP (1  $\mu$ M) for 1 h at room temperature prior to incubation with the membranes. Following the incubation, the membranes were washed with TBS containing 0.05% Tween 20 and exposed to Kodak BioMax MR film (Eastman Kodak Company, Rochester, NY) at room temperature.

For non-radioactive probing, filters were incubated with unlabeled LRP-2 (1 nM) in blocking buffer containing 5 mM CaCl<sub>2</sub> and 0.05% Tween 20 for 3 h at room temperature. Bound receptor was detected using mouse anti-LRP-2 IgG (1  $\mu$ g/ml), anti-mouse IgG-HRP, and the ECL Plus Western blotting detection system (Amersham Pharmacia Biotech).

**Protein Sequence Analysis**—Seminal vesicle fluid, isolated in the presence of spermine and spermidine, was electrophoretically separated on a 4–12% acrylamide gel in the presence of SDS, under non-reducing conditions. The gel was stained with 0.2% Coomassie Brilliant Blue R-250 (Bio-Rad) in 45% methanol, 10% acetic acid. The polypeptide band having an *M<sub>r</sub>* corresponding to that identified in seminal fluid by gel-blot overlay assay using radioiodinated LRP-2 as probe was excised. The gel slice was minced, and the pieces were washed and destained in 50% methanol for 18 h. The gel pieces were dehydrated in acetonitrile and rehydrated in 10 mM dithiothreitol, 0.1 M ammonium bicarbonate and reduced at 55 °C for 1 h. The dithiothreitol solution was removed, and the sample was alkylated in 50 mM iodoacetamide, 0.1 M ammonium bicarbonate for 1 h at room temperature in the dark. The gel pieces were washed with 0.1 M ammonium bicarbonate and dehydrated in acetonitrile for 5 min. The acetonitrile was removed, and the gel slices were rehydrated in 0.1 M ammonium bicarbonate. The pieces were again dehydrated in acetonitrile and dried by vacuum centrifugation. The gel pieces were rehydrated in a solution of trypsin (12.5 ng/ $\mu$ l) in 0.1 M ammonium bicarbonate and incubated on ice for 45 min. The trypsin-containing solution was removed, 0.05 M ammonium bicarbonate was added, and the slices were incubated for 18 h at 37 °C. The proteolytic fragments were then extracted using 50% acetonitrile, 5% formic acid and dried to <20  $\mu$ l. The sample was then analyzed by capillary high performance liquid chromatography-electrospray tandem mass spectrometry using a Finnigan-MAT TSQ7000 located in the W. M. Keck Biomedical Mass Spectrometry Laboratory (University of Virginia, Charlottesville, VA).

**Solid Phase Binding Assay**—Both homologous and heterologous ligand displacement assays were carried out as described previously (25). Radioiodinated proteins (1–2 nM) in TBS, 3% bovine serum albumin (U. S. Biochemical Corp.), 5 mM CaCl<sub>2</sub>, 0.05% Tween 20 were incubated for 3 h at room temperature in breakaway microtiter wells (Dynatech, Chantilly, VA) coated with LRP-2, SVS-II, or ovalbumin (each coated at 3  $\mu$ g/ml) in the presence of increasing concentrations of the competitor. The wells were washed with TBS, 5 mM CaCl<sub>2</sub>, 0.05% Tween 20, and bound radioactivity was determined using a gamma counter. The computer program Ligand (26) was used to analyze the competition data and to determine dissociation constants (*K<sub>d</sub>*) for receptor-ligand interactions.

**Assay For Cellular Internalization and Degradation of <sup>125</sup>I-SVS-II**—Ligand internalization and degradation by LRP-2 expressing cells was performed according to previously published methods (24, 27). Briefly, RA- and Bt<sub>2</sub>cAMP-treated F9 cells were plated into 3.83-cm<sup>2</sup> wells at 1.5–1.75  $\times$  10<sup>6</sup> cells/well and allowed to grow for 18 h at 37 °C, 5% CO<sub>2</sub>. Prior to the addition of radioactive ligands, the cells were washed with DMEM and incubated in the assay medium (DMEM, 20 mM HEPES, 1% Nutridoma serum substitute (Boehringer Mannheim), penicillin/streptomycin, and 1.2% bovine serum albumin) containing either SVS-II (1  $\mu$ M), RAP (1  $\mu$ M) or IgGs (200  $\mu$ g/ml) for 0.5 h at 37 °C, 5% CO<sub>2</sub>. To inhibit the lysosomal protease activity, the cells were pre-treated with 0.1 mM chloroquine (Sigma) for 0.5 h at 37 °C, 5% CO<sub>2</sub>. The

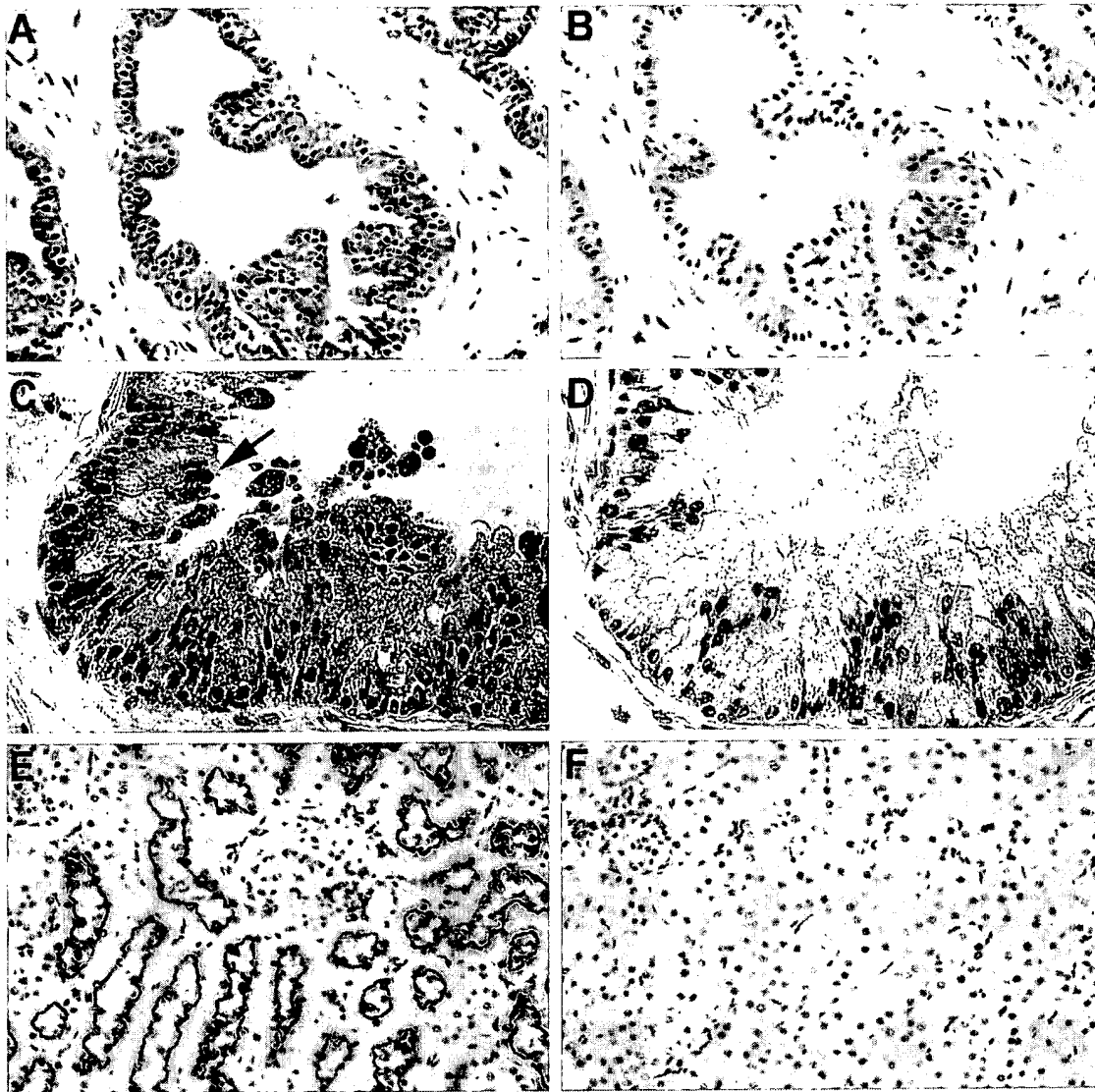


FIG. 1. Immunohistochemical localization of LRP-2 within rat seminal vesicle epithelial cells located proximal to the duct. Rat seminal vesicle (panels A-D) and rat kidney tissue sections (panels E-F) were incubated with affinity purified rabbit anti-LRP-2 IgG (panels A, C, and E) or pre-immune rabbit IgG (panels B, D, and F) followed by goat anti-rabbit-horseradish peroxidase and the chromogenic substrate DAB. The tissue sections were counter-stained using hematoxylin.

pre-incubation medium was removed, and medium containing the radioiodinated proteins (1 nM) plus competitors was added and incubated for 5 h at 37 °C, 5% CO<sub>2</sub>. The conditioned culture medium was treated with trichloroacetic acid (final concentration 10%) and centrifuged at 10,000 × *g* for 10 min. The amount of radioactivity present in the supernatant was taken to represent the amount of degraded SVS-II (28). The cell layer was washed three times with cold (4 °C) Dulbecco's phosphate-buffered saline and then treated with 0.5 mg/ml trypsin, 50 µg/ml proteinase K, and 0.5 mM EDTA (all from Sigma) in Dulbecco's phosphate-buffered saline for 2–4 min at 4 °C. The released cells were pelleted by centrifugation at 1,300 × *g* for 15 min, and the amount of radioactivity in the cell pellet was measured.

#### RESULTS

**Immunohistological Analysis Reveals that LRP-2 Is Expressed by Seminal Vesicle Epithelial Cells Located Proximal to the Duct**—We have recently found that LRP-2 was expressed on apical surfaces of epithelial cells lining the efferent ducts and in the intermediate zone, proximal head, corpus, and caudal regions of the epididymis (2). In the present study, we have examined LRP-2 expression in the accessory sex gland, the seminal vesicle. As shown in Fig. 1, using affinity-selected LRP-2 antibodies, pronounced immunoperoxidase staining was observed on the apical regions of epithelial cells covering the duct of the gland. In addition, LRP-2 staining was also strong

in the apical portion of the epithelial cells lining the ampulla (Fig. 1C). This staining also appeared to occur in budding vesicles (Fig. 1C, arrow). Very weak LRP-2 staining was seen in the underlying stroma lying adjacent to the LRP-2-expressing epithelial cells. Examination of distal segments of the gland showed negligible LRP-2 staining in both the epithelial cells and the underlying stromal cells (data not shown). The results indicate that LRP-2 is expressed by epithelial cells lining the duct of the seminal vesicles and may be present within secreted vesicular particles.

**LRP-2 Binds to a 100-kDa Protein Present in Seminal Vesicle Fluid**—The expression of LRP-2 in the seminal vesicle prompted us to examine seminal vesicle fluid for the presence of LRP-2-binding proteins. Seminal vesicle fluid collected in the presence of the polyamines spermine and spermidine, to prevent coagulation, was electrophoretically separated by SDS-polyacrylamide gel electrophoresis (under non-reducing conditions) and transferred to nitrocellulose membranes. The membrane-bound proteins were probed using <sup>125</sup>I-LRP-2. As shown in Fig. 2, the radiolabeled receptor bound to a polypeptide with an *M<sub>r</sub>* of 100,000 (Fig. 2A). Other minor LRP-2-binding bands were also apparent in the profile. As a control, the <sup>125</sup>I-LRP-2 was shown to bind to a known LRP-2 ligand, the

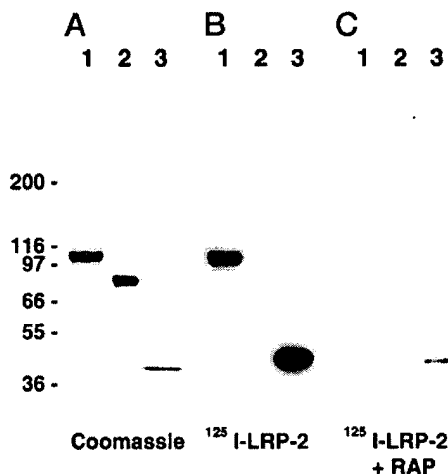


FIG. 2. Radiolabeled LRP-2 binds to a 100-kDa polypeptide present in seminal vesicle fluid. Rat seminal vesicle fluid proteins (7  $\mu$ g; lane 1), human fibulin-1 (3  $\mu$ g; lane 2), and human RAP (1  $\mu$ g; lane 3) were electrophoretically separated on SDS-containing 4–12% polyacrylamide gradient gels in the absence of reducing agent. Separated proteins were either stained with Coomassie Blue (A) or transferred to nitrocellulose membranes and probed with  $^{125}$ I-LRP-2 (1 nM; B) and  $^{125}$ I-LRP-2 plus RAP (1  $\mu$ M), an antagonist of the ligand binding activity of LRP-2 (C).

39-kDa RAP (29–31). Fibulin-1, a non-LRP-2-binding protein did not exhibit binding to the radiolabeled receptor in this assay.

To evaluate the specificity of the binding interaction between the receptor and the 100-kDa protein, duplicate filters were incubated with  $^{125}$ I-LRP-2 in the presence of RAP, an antagonist of the ligand binding activity of LRP-2 (19). As shown in Fig. 2C, RAP completely blocked binding of radioiodinated LRP-2 to the 100-kDa protein. The results indicate that seminal vesicle fluid contains a 100-kDa protein that binds to LRP-2. Consistent with all other LRP-2 ligands (19), the binding of the 100-kDa protein to LRP-2 was inhibited by RAP.

**Identification of the 100-kDa LRP-2-binding Protein as Seminal Vesicle Secretory Protein II by Protein Sequence Analysis**—To identify the 100-kDa LRP-2-binding protein, amino acid sequence analysis was performed. Initial attempts to sequence the N terminus of the LRP-2-binding protein using Edman degradation did not yield a sequence. Assuming that the protein might be N-terminal blocked, it was subjected to limited proteolysis, and the resulting fragments were evaluated by liquid capillary mass spectrometry and high performance liquid chromatography-tandem mass spectrometry. As a result, sequences were obtained from 13 peptides (Table II). Data base analysis of these sequences revealed that eleven corresponded to sequences contained within rat seminal vesicle secretory protein (SVS-II) (32). SVS-II is a 48-kDa seminal fluid protein that forms a 100-kDa disulfide-linked homodimer (16, 17, 32). Sequences from two of the peptides were unidentifiable. The results indicate that the 100-kDa LRP-2-binding protein most likely corresponds to SVS-II.

**SVS-II Binds with High Affinity to LRP-2 in Solid Phase Assay**—To confirm that LRP-2 binds to SVS-II, solid phase binding assays were performed using purified components. SVS-II was purified from rat seminal vesicle fluid according to previously published methods (16). As shown in Fig. 3A,  $^{125}$ I-LRP-2 bound to purified SVS-II immobilized on microtiter well plastic but not to ovalbumin-coated wells. The binding to SVS-II could be inhibited by incubation with unlabeled LRP-2. A dissociation constant ( $K_d$ ) of  $10.4 \pm 4.9$  nM ( $n = 6$ ) was determined for the binding of radiolabeled LRP-2 to SVS-II from the best fit of the data to a single class of binding sites model. Similarly,  $^{125}$ I-SVS-II was shown to bind to wells coated

with LRP-2, but not to ovalbumin-coated wells (Fig. 3B). A  $K_d$  of  $5.8 \pm 2.5$  nM ( $n = 5$ ) was determined for the binding of SVS-II to immobilized LRP-2. The differences in the observed  $K_d$  values derived from the two types of assays may reflect conformational differences between the solution phase and plastic-absorbed proteins. In addition, the LRP-2 antagonist RAP was shown to competitively inhibit  $^{125}$ I-SVS-II binding to LRP-2-coated wells (Fig. 3C), and an inhibition constant ( $K_i$ ) of  $9.0 \pm 2.5$  nM ( $n = 4$ ) was determined. The findings indicate that LRP-2 can bind to SVS-II with high affinity and that the interaction can be blocked by RAP.

Given that LRP-2 shares a number of ligands in common with LRP-1, we were interested in evaluating the ability of LRP-1 to bind to SVS-II. As shown in Fig. 4A,  $^{125}$ I-LRP-1 bound to purified SVS-II immobilized on microtiter well plastic, and the binding could be inhibited by incubation with solution phase SVS-II. Additionally,  $^{125}$ I-SVS-II was found to bind to wells coated with LRP-1 but not to ovalbumin-coated wells (Fig. 4B). A  $K_d$  of  $1.4 \pm 1.0$  nM ( $n = 3$ ) was determined for the binding of SVS-II to immobilized LRP-1. RAP was found to also inhibit the binding of SVS-II to immobilized LRP-1 ( $K_i = 4.4 \pm 0.6$  nM) (Fig. 4C). A  $K_i$  of  $16.5 \pm 3.5$  nM ( $n = 2$ ) was determined for SVS-II inhibition of binding of radiolabeled LRP-1 to immobilized SVS-II. The results indicate that LRP-1, like LRP-2 can bind to SVS-II *in vitro*; however, given that LRP-1 was not immunologically detected in the seminal vesicle (data not shown), the significance of this interaction remains uncertain.

**LRP-2 Binds to a Site within the 13-Residue Repeat Region of SVS-II**—To determine the binding site within the SVS-II protein, a series of fragments of SVS-II were generated recombinantly and evaluated for their ability to promote LRP-2 binding in gel-blot overlay assays. The recombinant fragments corresponding to the 85-residue N-terminal domain, the 103-residue C-terminal domain, or the 204-residue 13-residue repeat domain of SVS-II (Fig. 5) were expressed as fusions with GST. As shown in Fig. 6, LRP-2 bound the 204-residue 13-residue-repeat domain-containing fragment but not the N- and C-terminal domain fragments. As controls, LRP-2 bound filter-immobilized SVS-II and RAP, but not GST. LRP-2 binding to SVS-II, the 204-residue 13-residue-repeat domain-containing fragment and RAP was inhibited by RAP (Fig. 6, C).

**LRP-2 Mediates Cellular Uptake and Lysosomal Degradation of SVS-II**—To determine whether LRP-2 functions to mediate endocytosis leading to lysosomal degradation of SVS-II, we evaluated the ability of LRP-2-expressing cells to internalize and degrade radioiodinated SVS-II. As shown in Fig. 7, cultured F9 cells, treated to augment expression of LRP-2 (24, 27) mediated internalization and degradation of  $^{125}$ I-SVS-II. Both processes could be inhibited by polyclonal LRP-2 antibodies that have been previously shown to block LRP-2 function (24, 27, 33). Control IgGs had no effect on  $^{125}$ I-SVS-II internalization and degradation. The magnitude of the inhibition by LRP-2 antibodies (200  $\mu$ g/ml) was nearly as great as that obtained using either 1000-fold molar excess of unlabeled SVS-II or RAP as competitors. The degradation of radiolabeled SVS-II was inhibited by chloroquine (Fig. 7A), a drug that blocks lysosomal proteinase activity through its ability to increase the pH of lysosomal vesicles. As expected, the chloroquine treatment resulted in an intracellular accumulation of  $^{125}$ I-SVS-II (Fig. 7B). The findings indicate that LRP-2 can function to mediate endocytosis and lysosomal degradation of  $^{125}$ I-SVS-II.

## DISCUSSION

Evidence is presented in this study to indicate that LRP-2, a multi-ligand endocytic receptor and a member of the LDL receptor family, is expressed by seminal vesicle epithelial cells

TABLE II

Mass spectroscopic sequence analysis of trypsin-generated proteolytic fragments of 100-kDa seminal vesicle fluid protein

X designates isoleucine or leucine which cannot be distinguished by low energy collisionally activated dissociation; M(o) designates oxidized methionine; lowercase letters designate tentative assignments; \_ designates an unknown amino acid; - - - designates an unknown number of unknown amino acids; b2 = refers to the mass (in Da) of the first two amino acid residues; UI indicates that the peptide sequence obtained did not correspond to any sequence in the database.

Peptide number	Measured molecular mass	Peptide sequence by collisionally activated dissociation	Peptide sequence of SVS-II from data base	Amino acid position within rat SVS-II
	Da	calculated molecular mass, Da	calculated molecular mass, Da	
1	697.8	SFGQMK	SFGQMK (697.8)	184-189
2	870.5	_XAQYR (b2 = 221)	GYLAQYR (871.0)	376-382
3	886.6	_XNFGXK (b2 = 195)	GHLNFGK (886.0)	44-51
4	1052.8	SFDQDAQXK	SFDQDAQLK (1052.8)	271-279
5	1055.0	SYGEETQXK	SYGEETQLK (1055.1)	262-270
6	1106.2	_AGXYQAQXK	GGAGLYQAQLK (1106.2)	403-413
7	1163.0	NAGXYQAQXGK (1163.3)	U.I.	
8	1183.4	QSYx - - - GXK	U.I.	
9	1274.8	_NQDNFFTK (b2 = 262)	NFNQDNFFTK (1275.4)	393-402
10	1368.6	_AMDEDXSQVR (b2 = 205)	GFAMDEDLSQVR (1368.5)	322-333
11	1384.6	_AM(o)DEDXSQVR (b2 = 205)	GFAM(o)DEDLSQVR (1384.5)	322-333
12	1428.2	_GGQXQSYGQM(o)	SQGGQXQSYGQM(o)	216-228
13	1439.0	TEEDXSQFGQQR	TEEDLSQFGQQR	353-364

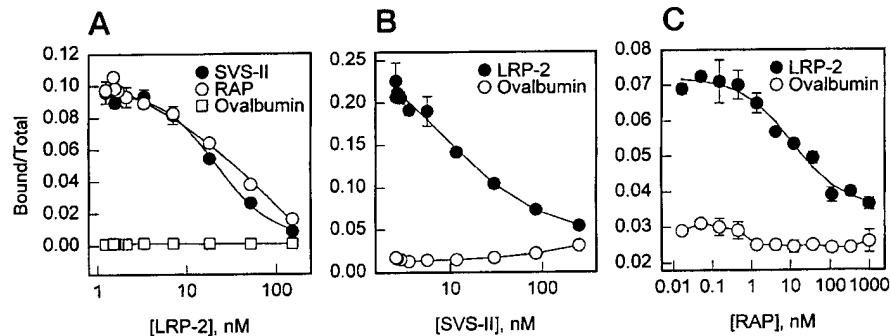


FIG. 3. Homologous and heterologous ligand displacement assays demonstrate the ability of solution-phase and immobilized LRP-2 to bind SVS-II. Panel A shows  $^{125}\text{I}$ -LRP-2 binding to purified SVS-II immobilized on microtiter well plastic. The binding to SVS-II could be inhibited by incubation with unlabeled LRP-2. A dissociation constant ( $K_d$ ) of  $10.4 \pm 4.9$  nM ( $n = 6$ ) was determined after fitting the data using a single class of binding sites model and the computer program Ligand. Panel B shows  $^{125}\text{I}$ -SVS-II binding to wells coated with LRP-2, and a  $K_d$  of  $5.8 \pm 2.5$  nM ( $n = 5$ ) was determined for the binding interaction. In panel C, the LRP-2 antagonist RAP was used to competitively inhibit  $^{125}\text{I}$ -SVS-II binding to LRP-2-coated wells, and an inhibition constant ( $K_i$ ) of  $9.0 \pm 2.5$  nM ( $n = 4$ ) was determined.

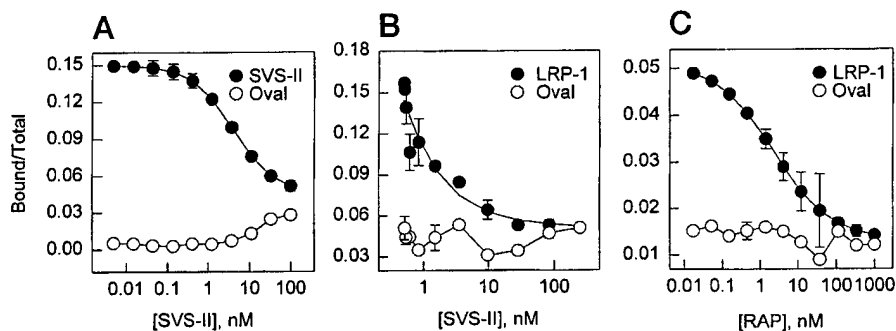


FIG. 4. Homologous and heterologous ligand displacement assays demonstrate the ability of solution phase and immobilized LRP-1 to bind SVS-II. Panel A shows  $^{125}\text{I}$ -LRP-1 binding to purified SVS-II immobilized on microtiter-well plastic. The binding of LRP-1 to SVS-II could be inhibited by incubation with unlabeled SVS-II. An inhibition constant ( $K_i$ ) of  $16.5 \pm 3.5$  nM ( $n = 2$ ) was determined after fitting the data using a single class of binding sites model and the computer program Ligand. Panel B shows  $^{125}\text{I}$ -SVS-II binding to wells coated with LRP-1, and a dissociation constant ( $K_d$ ) of  $1.4 \pm 1.0$  nM ( $n = 3$ ) was determined for the binding interaction. In panel C, the LRP-1 antagonist RAP was used to competitively inhibit  $^{125}\text{I}$ -SVS-II binding to LRP-1-coated wells, and an inhibition constant ( $K_i$ ) of  $4.4 \pm 0.6$  nM ( $n = 2$ ) was determined.

and is potentially a component of seminal vesicle-secretory vesicles. In addition, it was discovered that LRP-2 binds with high affinity ( $K_d = 5.6$  nM) to SVS-II, a prominent seminal vesicle-derived coagulatory protein. The LRP-2 binding site was mapped to a 204-amino acid region of the protein contain-

ing a series of 13-residue repeats. As evidence that LRP-2 is also capable of mediating cellular clearance of SVS-II, LRP-2-expressing cells were shown to endocytose and degrade SVS-II. Both processes can be inhibited by anti-LRP-2 IgG or RAP, a known antagonist of LRP-2 function (23, 24, 33, 34).

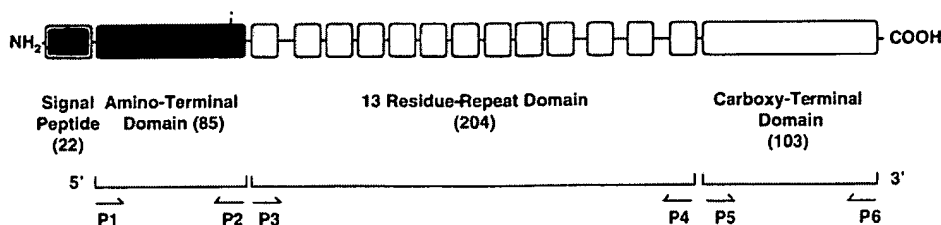


FIG. 5. Schematic diagram depicting the domain organization of rat SVS-II. Sizes of individual domains in amino acid residues are indicated in parentheses. The locations of oligonucleotide primers described in Table I for PCR amplification of cDNAs encoding regions of SVS-II are indicated. Asterisk indicates the position of the cysteine residue presumably involved in disulfide bond-stabilized dimer formation.

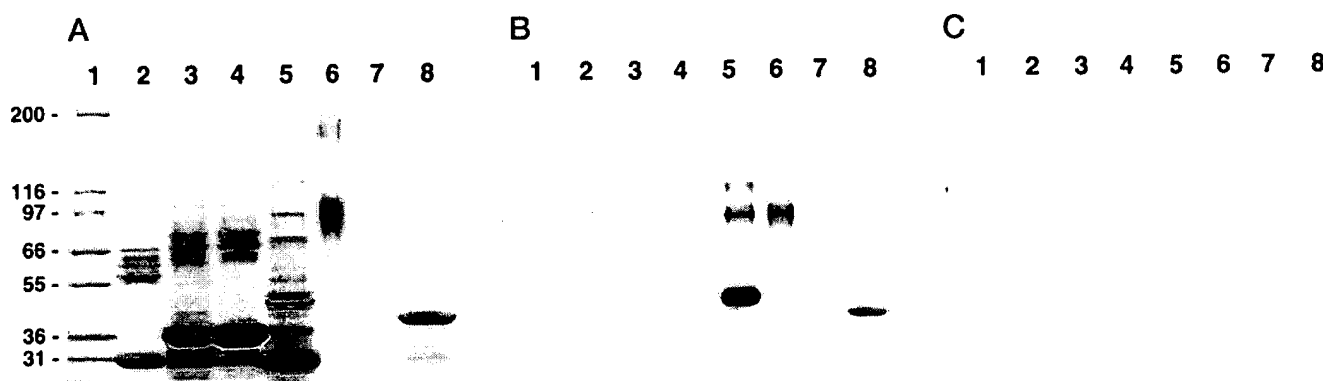
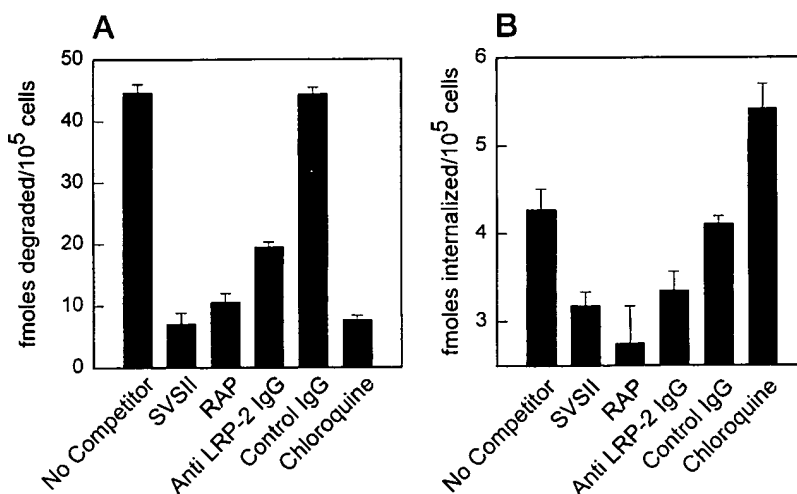


FIG. 6. LRP-2 binds to a central 13-residue-repeat domain within SVS-II. Shown in panel A is a Coomassie-stained 4–12% polyacrylamide gradient gel containing molecular weight standards, (lane 1), GST (1  $\mu$ g; lane 2), SVS-II N-terminal domain-GST fusion protein (1  $\mu$ g; lane 3), SVS-II C-terminal domain-GST fusion protein (1  $\mu$ g; lane 4), SVS-II 13-residue-repeat domain-GST fusion protein (1  $\mu$ g; lane 5), rat SVS-II protein (1  $\mu$ g; lane 6), and RAP (1  $\mu$ g; lane 8) electrophoretically separated in the absence of reducing agent. Panels B and C show autoradiographs of a membrane containing proteins from duplicate gels that were transferred to PVDF membranes and probed with LRP-2 (1 nM; panel B) or LRP-2 plus RAP (1  $\mu$ M) and detected with monoclonal anti-LRP-2 antibody. Indicated on the left are molecular mass values of the marker proteins in kDa.

FIG. 7. LRP-2 antibodies and RAP inhibit endocytosis and lysosomal degradation of  $^{125}$ I-SVS-II by LRP-2-expressing cells. RA/Bt<sub>2</sub>cAMP-differentiated F9 cells were incubated with  $^{125}$ I-SVS-II (1 nM) in the presence of unlabeled SVS-II (1  $\mu$ M), RAP (1  $\mu$ M), affinity purified anti-LRP-2 IgG (200  $\mu$ g/ml), control rabbit IgG (200  $\mu$ g/ml), or chloroquine (0.1 mM) for 5 h at 37  $^{\circ}$ C, 5% CO<sub>2</sub>. Shown are the amounts of  $^{125}$ I-SVS-II internalized (B) and degraded (A) by the cells. Plotted values are means  $\pm$  S.D. of triplicate values and are representative of duplicate experiments.



The physiological significance of the LRP-2 interaction with SVS-II is not yet known. LRP-2-mediated endocytosis of SVS-II may function *in vivo* to clear multimerized and/or transglutaminase-cross-linked SVS-II, thereby preventing coagulation within the male reproductive tract and the urinary tract which might lead to an obstruction. Proteinaceous plugs have been reported in the accessory sex glands and urinary bladders of male rats (35, 36). The expression of LRP-2 in epithelial cells proximal to the ductal region of the gland might be important to clear SVS-II that has become cross-linked by retrograde flow of transglutaminase-containing fluids derived from the coagulatory and prostate glands. LRP-2 that is expressed by epithelial cells lining the female reproductive tract (1, 4, 37) might also mediate clearance of proteolytic fragments of SVS-II generated during dissolution of the coagulatory plug. Indeed, following copulation, seminal vesicle antigens have been detected

within uterine epithelial cells, indicative of endocytosis as a mechanism of their elimination (38).

Immunohistological staining of the seminal vesicle not only showed LRP-2 to be localized within the epithelial cells lining the ductal region of the gland, but possibly in membrane bound vesicular bodies found in the lumen of the gland. While LRP-2-expressing epithelial cells lacking apparent vesicular budding were observed, numerous regions of epithelium in the ductal area appeared to be active in secretion of LRP-2-containing vesicular bodies. The pronounced LRP-2 staining of the budding vesicles in these areas may indicate that LRP-2 serves a role in vesicle secretion. Vesicular bodies have been observed by electron microscopy in the lumen of the seminal vesicle and other reproductive organs including the epididymis, vas deferens and ampulla (39). In addition, such vesicular bodies have been isolated from seminal vesicle (39–41), epididymal (42),

43), and prostatic fluids (44, 45). Following our previous finding of LRP-2 expression by epididymal epithelial cells (2), we isolated vesicular bodies from rat cauda epididymal fluid and found that they contained LRP-2 (unpublished observations, Ranganathan and Argraves). Vesicular bodies in seminal and epididymal fluids have been speculated to play a role in sperm maturation, perhaps involving proteolytic and glycolytic modification of spermatozoa membrane glycoproteins (46–48). It remains to be determined what role is served by LRP-2 associated with seminal and epididymal vesicular bodies.

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